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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number
WO 00/75176 A1

(51) International Patent Classification[7]: C07K 7/50, 7/56, 7/64, A61K 38/12, 38/08, 38/16, A61P 25/28

(21) International Application Number: PCT/AU00/00641

(22) International Filing Date: 7 June 2000 (07.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PQ 0848 8 June 1999 (08.06.1999) AU

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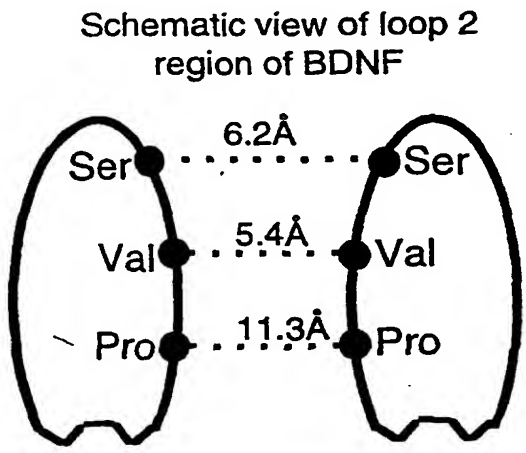
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European

[Continued on next page]

(54) Title: SMALL CYCLIC MIMICS OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

(57) Abstract: This invention relates to methods and compositions for promoting nerve cell growth and in particular to agonists of brain-derived neurotrophic factor. More specifically, the present invention relates to cyclic compounds comprising one or more cyclic moieties, which has a biological activity of brain-derived neurotrophic factor (BDNF).



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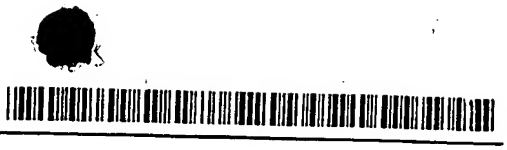


patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *With international search report.*

SMALL CYCLIC MIMICS OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

Field of the Invention

This invention relates to methods and compositions for promoting nerve cell growth and in particular to agonists of brain-derived neurotrophic factor. The invention relates more particularly to agonists which are derivatives of peptides based on the structures of the solvent-exposed loops 2 and 4 of brain-derived neurotrophic factor.

Background of the Invention

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of neurotrophic factors, which includes nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (Thoenen, 1991; Götz *et al*, 1994).

These factors promote the survival of neurons during embryonic development, and thus play a vital role in shaping the vertebrate nervous system. Between them, the neurotrophins support the survival of a wide range of peripheral and central neurons, although each individual neurotrophin acts on specific neuronal populations (for review see Thoenen, 1991; Götz *et al*, 1994).

In a variety of *in vitro* and *in vivo* models, BDNF has been shown to promote neuronal survival during embryonic development, and to prevent neuronal degeneration resulting from disease or injury. Furthermore, several BDNF-responsive neuronal populations have been implicated in human neurodegenerative disease. For example, in the central nervous system BDNF acts as a potent neurotrophic factor for cranial and spinal motor neurons which degenerate in amyotrophic lateral sclerosis (Thoenen *et al*, 1993), as well as for dopaminergic neurons of the substantia nigra which are lost in Parkinson's disease (Spina *et al*, 1992). In the periphery, BDNF has n urotrophic actions on small fibre sensory neurons

involved in several types of sensory neuropathy (Lindsay, 1994).

The biological effects of BDNF and the other neurotrophins are mediated by binding to two classes of cellular receptor: members of the trk family of receptor tyrosine kinases, and the low affinity neurotrophin receptor, p75. Specific neurotrophins bind with high affinity (K_d approximately 10^{-11} M) to particular trk members expressed by responsive neurons: thus NGF and NT-3 bind to trkA; BDNF and NT-4/5 bind to trkB; NT-3 binds to trkC. Binding of a neurotrophin to its specific-trk receptor causes receptor homodimerisation, triggering the intrinsic kinase domains of the receptors to autophosphorylate intracellular tyrosine residues, and thus initiating signal transduction cascades leading to neuronal survival (Barbacid, 1994). In contrast, p75 acts as a common low affinity receptor for the neurotrophins, and binds each with comparable affinity (K_d approximately 10^{-9} M); p75 is expressed widely on central and peripheral neurons as well as on other cell types, such as Schwann cells (for review see Chao and Hempstead, 1995).

While the role of the trk members in signalling the neurotrophic effects of the neurotrophins is well established, the function of p75 remains controversial. Although there is compelling evidence that p75 either modulates responses mediated by trk members or itself plays a part in survival signalling, the final effect of p75 appears to depend on the relative levels of expression of p75 and trk (Kaplan and Miller, 1997). Of particular interest are the observations that p75 may, under certain circumstances, cause apoptosis either in the absence (Rabizadeh *et al*, 1993; Barrett and Bartlett, 1994) or presence (Frade *et al*, 1996) of bound neurotrophin. This "death signal" of p75 may be mediated by an intracellular region homologous to the death-signalling domains of tumour necrosis factor (TNF) receptor-1 and Fas (Chapman, 1995).

The neurotrophins are homodimers which consist of

two identical protomers of approximately 120 amino acids, held together by hydrophobic interactions. The overall amino acid homology between the different neurotrophins is approximately 50%, and sequence alignment between the members reveals a common pattern of sequence homology and variability (Ibáñez *et al*, 1993). X-ray crystal structures have been determined for the NGF homodimer (McDonald *et al*, 1991) and for a BDNF/NT-3 heterodimer (Robinson *et al*, 1995), revealing a common fold for the neurotrophins. This structure is depicted in Figure 1. In the neurotrophin protomer, the regions of high sequence homology exist as seven β-strands, which contribute to three longitudinal anti-parallel twisted β-sheets. This structure is locked by a "cystine knot" of three disulphide bridges. The six cysteine residues which participate in the cystine knot structure are fully conserved in all the neurotrophins. The three pairs of β-strands are linked by three β-hairpin loops (loop 1, loop 2 and loop 4) and a longer loop (loop 3), which correspond predominantly to the regions of sequence variability.

It has been postulated that the β-hairpin loop regions of the neurotrophins are responsible for the specificity of different trk receptors, and thus are important regions in receptor binding and activation. In general, structure-activity data obtained from the neurotrophins support this hypothesis. Site-directed mutagenesis studies have identified amino acid residues of in loop 2 of BDNF, which are important for binding to trkB and for biological activity. Insertion of this region of BDNF into NGF gave a chimeric protein which, unlike native NGF bound to trkB and displayed BDNF-like biological activity (Ibáñez *et al*, 1993). Additional residues in loop 3 (Gln^{84}) and loop 4 (Lys^{96} and Arg^{97}) have been shown to be important in activation of trkB, but are thought not to be involv d in receptor binding. When mapped on to the three-dimensional structure of BDNF, these residues are solvent-accessible, and together form a binding surface that almost

exclusively spans the top half of the molecule.

Other site-directed mutagenesis studies have shown that three positively charged residues in each of the neurotrophins are of paramount importance in binding to p75 (Ibáñez *et al*, 1992; Rydén *et al*, 1995). These data are consistent with the idea that p75 shares a common binding interface with the neurotrophin family. There are, however, differences in the position of these three residues in different neurotrophins: in NGF, NT-3 and NT-4/5, the three positively-charged residues are spread across two adjacent loops, while in BDNF the three positively-charged residues are contiguous amino acids (Lys^{95}-Lys^{96}-Arg^{97}), located on loop 4 (Figure 1).

The ability of exogenously administered neurotrophic factors such as BDNF to rescue neurons in a variety of *in vivo* models of neurodegeneration has led to the widespread belief that neurotrophins and other neurotrophic factors offer exciting prospects for the treatment of neurodegenerative diseases, such as motor neuron disease and peripheral neuropathies (for review see Hefti, 1994). Unfortunately, because they are proteins, neurotrophic factors are orally inactive, are unable to cross the blood-brain barrier, and typically have a short half-life in plasma (Dittrich *et al*, 1994). Thus the recombinant human neurotrophic factors themselves are unlikely to be optimal agents for the long-term treatment of neurodegenerative disease. Indeed, the lack of success thus far of neurotrophic factors in clinical trials for the treatment of motor neuron disease has been attributed to the inability of the proteins to reach their targets in the central nervous system (CNS) following subcutaneous administration (Penn *et al*, 1997). One means of circumventing these problems would be to develop low molecular weight, non-peptidic analogues of neurotrophic factors with improved pharmacokinetic charact ristics.

For example, Australian Patent Application No. 24264/97 by Regents of the University of California, and

Longo *et al*, 1997, disclose low molecular weight compounds, 8-9 amino acids long and incorporating a D-penicillamine group, which bind to the p75 region of the NGF receptor, and promote neuronal survival in primary cultures of chick dorsal root ganglia. The compounds form undefined (either parallel or anti-parallel) monocyclic dimers of 16-18 amino acids. International Patent Application No. WO95/15593 by Queen's University at Kingston discloses bicyclic peptides based on the cysteine knot region, which is distal from loops 1-4 of BDNF; these bicyclic peptides act as neurotrophin antagonists in the chick dorsal root ganglion assay. Cyclic peptides based on various loop regions of NGF have been shown to interfere with NGF-mediated biological activity (LeSauter *et al*, 1995).

We have used a model of the three-dimensional structure of BDNF to design small, conformationally-constrained peptides that mimic the receptor-binding loops of BDNF. These peptides have been synthesised and purified, and then assayed in cultures of embryonic chick sensory neurons, a subpopulation of which require BDNF for survival.

Monomeric cyclic peptides designed in this mann r from loop 2 - believed to be the major region of BDNF contributing to trkB binding and activation - act as specific antagonists of BDNF-mediated neuronal survival in cell culture. We have investigated structure-activity relationships in these peptides by conducting an alanine scan, and have used pharmacodynamic simulations to model the anticipated competitive mode of antagonism of these peptides (O'Leary and Hughes, 1998).

We have now used the structure-activity data obtained for these monomeric cyclic peptides to design bicyclic dimeric peptides with BDNF agonist activity. The peptides consist of two monocyclic peptides connected by a linking moiety. It is surprising that the linking moiety in some of the dimers found to be active is incorporated at sites other than those predicted to be optimal on th basis

of the structure-activity relationships. In addition, the linker distance found to give optimal activity was shorter than that predicted from our design template.

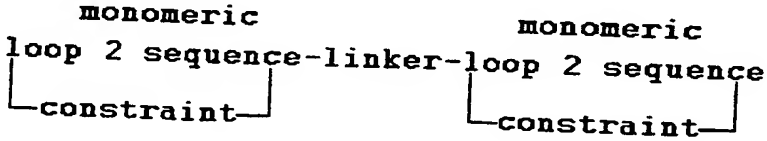
By combining these data, we have been able to create a novel class of tricyclic dimeric peptides with markedly improved potency over the dimeric peptides.

In contrast, monomeric cyclic peptides based on the p75-binding tripeptide sequence found in loop 4 had no inhibitory effects on the neuronal survival activity of either BDNF or NGF (Zwar and Hughes, 1997). To our surprise, however, when tested in the absence of neurotrophin some of these monomeric peptides acted as BDNF-like agonists, able to promote the survival of chick sensory neurons in culture.

Summary of the Invention

According to a first aspect, the invention provides a cyclic compound of one or more cyclic moieties, which has a biological activity of brain-derived neurotrophic factor (BDNF).

In one embodiment, the compound is a bicyclic dimeric compound (that is, a compound composed of two monocyclic compounds connected by a chemical linker) based on loop 2 of BDNF, of general formula (I):



(I)

wherein:

monomeric loop 2 sequence means a sequence of amino acid residues or functional equivalents thereof, which is substantially homologous to the loop 2 region of BDNF, and which comprises all or part of the following sequence:

Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51};

constraint means any chemically and biologically compatible grouping of atoms serving to limit the flexibility of the monomeric loop 2 sequence; and

linker means any chemically and biologically compatible grouping of atoms serving to link two monomeric loop 2 sequences and their associated constraints to give a bicyclic, dimeric compound.

Generally, the preferred linking groups have 0 to 20 carbon atoms, and 0 to 10 heteroatoms (N, O, S, P etc.), and may be straight chain or branched, may contain saturated, unsaturated and/or aromatic rings, may contain single and/or double bonds, and may contain chemical groups such as amide, ester, disulphide, thioether, ether, phosphate, amine and the like.

The "constraint" can be obtained by several methods, including but not limited to:

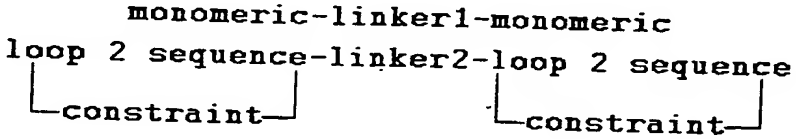
(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, for example by condensation with an ω-amino carboxylic acid;

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added on to the monomeric loop 2 sequence for this purpose; and,

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either dir ctly or a spac r group as described in (i) above. The residues contributing the side

chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added on to the monomeric loop 2 sequence for this purpose.

In a second embodiment, the compound is a tricyclic dimeric compound (that is, a compound composed of two monocyclic compounds connected by two chemical linkers) based on loop 2 of BDNF of general formula (II):



(II)

wherein:

monomeric loop 2 sequence means a sequence of amino acid residues or functional equivalents thereof, which is substantially homologous to the loop 2 region of BDNF, and which comprises all or part of the following sequence:

Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51};

constraint means any chemically and biologically compatible grouping of atoms serving to limit the flexibility of the monomeric loop 2 sequence; and

linker means any chemically and biologically compatible grouping of atoms serving to link two monomeric loop 2 sequences and their associated constraints to give a tricyclic, dimeric compound. These linkers may be the same or different.

Generally, the preferred linking groups have 0 to 20 carbon atoms, and 0 to 10 heteroatoms (N, O, S, P etc.), and may be straight or branched, may contain saturated, unsaturated and/or aromatic rings, may contain single and/or double bonds, and may contain chemical groups such as amide, ester, disulphide, thioether, ether, phosphate, amine and the like.

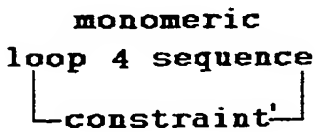
The "constraint" can be obtained by several methods, including but not limited to:

(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function either directly via an amide bond betw n the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, for example by condensation with an ω-amino carboxylic acid;

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the "monomeric loop 2 sequence" itself, or may be incorporated into or added onto the monomeric loop 2 sequence for this purpose; and,

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or via the intermediacy of a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added onto the monomeric loop 2 sequence for this purpose.

In third embodiment, the compound is a monomeric, monocyclic compound based on the p75-binding region of loop 4 of BDNF and incorporating a molecular spacer of the general formula (III):



wherein:

monomeric loop 4 sequence means a sequence of amino acid residues or functional equivalents thereof, which is substantially homologous to the p75-binding region

of loop 4 of BDNF, and comprises all or part of the following sequence:

Lys^{95}-Lys^{96}-Arg^{97}; and,

constraint' means any chemically and biologically compatible grouping of atoms serving to limit the flexibility of the monomeric loop 4 sequence. For example by covalently linking all or part of the "monomeric loop 4 sequence" to form a cyclic structure (ring).

The "constraint'" can be derived by several methods, including but not limited to:

(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, such as one or more additional amino acid residues, including α- and ω-amino carboxylic acid residues;

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the "monomeric loop 4 sequence" itself, or may be incorporated into or added on to the "monomeric loop 4 sequence" for this purpose; and

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 4 sequence itself, or may be incorporated into or added on to the monomeric loop 4 sequence for this purpose.

Sequences encompassing conservative substitutions of amino acids are within the scope of the invention,

provided that the biological activity is retained.

It is to be clearly understood that the compounds of the invention include peptide analogues, including but not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods. See for example Chorev and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson *et al*, 1993.

3. Compounds in which individual amino acids are replaced by analogous structures, for example *gem*-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

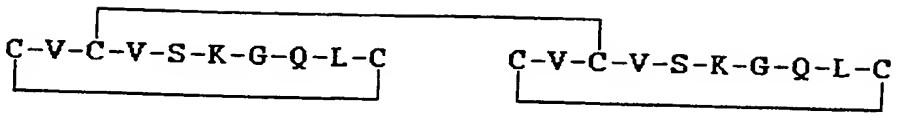
The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop *et al*, 1994). It is particularly contemplated that the compounds of the invention are useful as templates for design and synthesis of compounds of improved activity, stability and bioavailability.

Preferably where amino acid substitution is used, the substitution is conservative, *i.e.*, an amino acid is replaced by one of similar size and with similar charge properties.

In particularly preferred embodiments the bicyclic dimers are of formula (IV) to (VI):

$(L2\text{-}8P2C)_2$



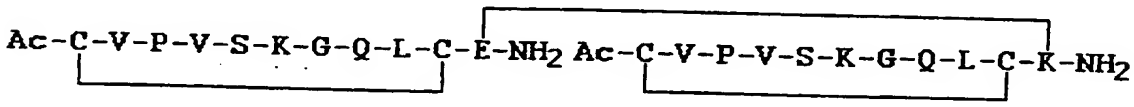
(IV)

$(L2\text{-}8S4C)_2$

(V)

where the dimeric bicyclic peptides $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ consist of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of a disulphide bond formed between cysteine residues substituted into the loop 2 sequence, or

$(L2\text{-}8\&E+K)_2$

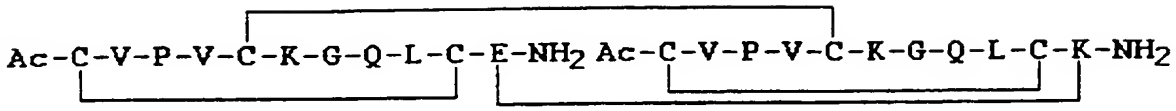


(VI)

where the dimeric bicyclic peptide $(L2\text{-}8\&E+K)_2$ consists of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of an amide bond formed between a glutamate and a lysine residue added to the loop 2 sequence.

In a particularly preferred embodiment the tricyclic dimer is of formula (VII):

$(L2\text{-}8S4C\&E+K)_2$



(VII)

wherein the dimeric tricyclic peptide $(L2\text{-}8S4C\&E+K)_2$ consists of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence, and joined by one linker consisting of a disulphide bond formed between cysteine residues substituted into the loop 2 sequence and a second substituted consisting of an amide bond formed between a glutamate and a lysine residue added to the loop 2 sequence.

In a particularly preferred embodiment the monomeric, monocyclic compound is of formula (VIII):

L4-3pA

(VIII)

wherein the monomeric cyclic compound is cyclised by condensing its amino- and carboxyl-termini directly via an amide bond.

The invention further provides a pharmaceutical composition, comprising a compound according to the invention together with a pharmaceutically acceptable carrier.

The composition may be formulated so as to be suitable for a variety of routes of administration, for example intravenous, subcutaneous, intramuscular or intrathecal or intraventricular injection, oral or for topical administration.

The exact formulation will depend on the individual route of administration. Methods and pharmaceutical carriers for preparation of pharmac utical

compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA. Pharmaceutically acceptable carriers include conventional carriers which are suitable for use with peptide-based drugs, including diluents, excipients, and preservatives and the like. For example, carriers such as dextrose, mannitol, sucrose, or lactose, buffer systems such as acetate, citrate and phosphate, and bulking agents such as serum albumin, preferably human serum albumin, may be used.

The invention also provides a culture medium additive for promotion of growth of neuronal cells *in vitro*, comprising a compound according to the invention together with a carrier or diluent which does not adversely effect the growth of cells in culture. Suitable carriers and diluents will be well known to the person skilled in art, and include physiologically acceptable fluids such as water, saline solution, or buffer solutions.

The optimal concentration of compound will vary according to the cell type and the culture conditions, but will generally be in the range 1-500μM, preferably 1-100μM.

The invention further provides a method of treatment of a condition characterised by neuronal deficit or neuronal death, comprising the step of administering an effective amount of a compound of the invention to a subject in need of such treatment.

It is contemplated that the method of the invention is suitable for treatment of conditions including but not limited to neurodegenerative diseases such as motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrome, Guamanian Parkinsonian dementia complex, progressive bulbar atrophy, Alzheimer's disease and the like, other neurodegenerative conditions such as those arising from ischaemia, hypoxia, neural injury,

surgery, exposure to neurotoxins such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and peripheral sensory neuropathies, including those resulting from exposure to drugs (such as cis-platin) and toxins and resulting from diabetes, for example mononeuropathy multiplex.

The dose required will depend on the nature and severity of the condition to be treated, and the route of administration, and will be at the discretion of the attending physician or surgeon. A suitable route, frequency of administration, and dosage can readily be established using conventional clinical trial methodology.

Throughout this specification, the amino acid numbering is the same as in mature BDNF, and conventional single-letter or three-letter amino acid code is used.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

The term "functional equivalents thereof", when used with reference to amino acid residues of the monomeric loop 2 or loop 4 sequence, means amino acid sequence variants of said sequence are encompassed. For example, one or more of the amino acids Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51} may be deleted, and optionally substituted by one or more amino acid residues; or wherein an amino acid residue has been covalently modified so that the resulting product is a non-naturally occurring amino acid. Amino acid sequence variants may be made synthetically, for example, by peptide synthesis, or may exist naturally.

An amino acid sequence variant of the monomeric loop 2 or loop 4 sequence of BDNF is included within the scope of the invention provided that it is functionally active. As used herein, "functionally active" and "functional activity" in reference to the monomeric loop 2 or loop 4 sequence of BDNF means that the compound generated therefrom is able to promote or enhance the

growth, survival, function, and/or differentiation of neurons and glia, especially axon fasciculation and process outgrowth, whether the neurons be central, peripheral, motorneurons, or sensory neurons, e.g. photoreceptors, vestibular ganglia, spinal ganglia and auditory hair cells. Therefore, monomeric loop 2 or loop 4 amino acid sequence variants generally will share at least about 75% (preferably greater than 80% and more preferably greater than 90%) sequence identity with the amino acid sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}, after aligning the sequences to provide for maximum homology, as determined, for example, by the Fitch, et al., *Proc. Nat. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman, et al., *J. Mol. Biol.* 48:443-453 (1970).

Amino acid sequence variants of the monomeric loop 2 or loop 4 sequence of BDNF are prepared by introducing appropriate amino changes into amino acid sequence, or by *in vitro* synthesis. Such variants include, for example, deletions from, or insertions or substitutions of, amino acid residues within Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}. Any combination of deletion, insertion, and substitution may be made to arrive at an amino acid sequence variant of the monomeric loop 2 or loop 4 sequence of BDNF, provided that such variant possesses the desired characteristics described herein.

There are two principal variables in the construction of amino acid sequence variants of the monomeric loop 2 or loop 4 sequence of BDNF: the location of the mutation site and the nature of the mutation. In general, the location and nature of the mutation chosen will depend upon the monomeric loop 2 or loop 4 sequence of BDNF characteristic to be modified.

For example, functionally active amino acid sequence variants of the monomeric loop 2 or loop 4 sequence of BDNF may be selected, for example, by substituting one or more amino acid residues in the amino

acid sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-L u^{49}-Lys^{50}-Gln^{51} with other amino acid r sidues of a similar or different polarity or charge.

One useful approach is called "alanine scanning mutagenesis." Here, an amino acid residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and, by means of recombinant DNA technology, replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Cunningham, et al., Science 244: 1081-1085 (1989). Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution.

Amino acid sequence deletions generally range from about 1 to 6 residues, more preferably about 1 to 3 residues, and typically are contiguous. Generally, the number of consecutive deletions will be selected so as to preserve the tertiary structure of the monomeric loop 2 or loop 4 sequence of BDNF.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions, or intrasequence insertions (i.e., insertions made within the amino acid sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3.

The third group of variants are those in which at least one amino acid residue in the amino acid sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}, preferably one to four, more preferably one to three, even more preferably one to two, and most preferably only one, has been removed and a different residue inserted in its place. The sites of greatest interest for making such substitutions are those sites most likely to be important to the functional activity of the monomeric loop 2 or loop 4 sequence of BDNF. Accordingly, to r tain

functional activity, those sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table A under the heading of preferred substitutions. If such substitutions do not result in a change in functional activity, then more substantial

Table A

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

changes, denominated exemplary substitutions in Table A, or as further described below in reference to amino acid classes, may be introduced and the resulting variant the monomeric loop 2 or loop 4 sequence of BDNF analyzed for functional activity.

Insertional, deletional, and substitutional changes in the amino acid sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51} may be made to improve the stability of the monomeric loop 2 or loop 4 sequence of BDNF.

Covalent modifications of the monomeric loop 2 or loop 4 sequence of BDNF are also included within the scope of this invention. For example, covalent modifications are introduced into the monomeric loop 2 or loop 4 sequence of BDNF by reacting targeted amino acid residues of the monomeric loop 2 or loop 4 sequence of BDNF with an organic derivatizing agent that is capable of reacting with selected amino acid side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethyl-pyro-carbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of

reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidat ; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatisation of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of

proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. Creighton, Proteins: Structure and Molecular Properties, pp.79-86 (W.H. Freeman & Co., 1983).

The term "substantially homologous" means that an amino acid sequence is quite similar to that of the monomeric loop 2 or loop 4 sequence of BDNF, and have at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the amino acids matching with at least 7 of the amino acids found in the sequence Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}.

The term "more resistant to metabolic degradation" means that the compound of the invention has been modified such that the resulting compound is more stable under acidic conditions than the "native" sequence of the monocyclic loop 2 or loop 4 sequence of BDNF. For example, amino acid substitutions as discussed previously may be undertaken which produce compounds more resistant to metabolic degradation. It is well known in the art that D-amino acids, and amino acids analogues are more resistant to acidic environments. Conjugates of small peptides and cholic acid have reduced metabolic degradation problems.

The term biological activity with reference to BDNF means a biological activity which is normally promoted, either directly or indirectly, by the presence of BDNF, and includes, but is not limited to, BDNF binding to the p75 receptor or the trkB receptor, neuron survival, neuron differentiation, including neuron process formation and neurite outgrowth, and biochemical effects such as induction of enzymes which are stimulated by BDNF. Such biological activities can be measured by conv ntional *in vitro* and *in vivo* assays, such as the chick dorsal root ganglion assay described herein by Barde *et al* (1980) and

the neurite outgrowth, and *in* vivo kindling assays described in WO97/15593 and by Riopelle *et al* (1982).

It will be clearly understood that the compounds of the invention may be synthesised by any suitable method. Solid phase methods such as those developed for synthesis of peptides and peptidomimetic compounds are preferred, including but not limited to the Fmoc solid phase peptide synthesis method described herein, the Boc solid phase peptide synthesis method, and PIN synthesis methods (for review, see Maeji *et al.*, 1995). Those skilled in the art will readily be able to select the most suitable method for any given compound of the invention.

Brief Description of the Figures

Figure 1 shows the backbone trace of the three-dimensional structure of BDNF dimer (one monomer in black, the other grey), showing the positions of the loop 2 (trkB binding) and loop 4 (predominantly p75 binding) regions. Side chains of the p75 binding tripeptide in loop 4 (Lys-Lys-Arg) are shown.

Figure 2 illustrates the molecular modelling of monomeric cyclic loop 2 analogues. An α-carbon to α-carbon trace of the native loop 2 of BDNF is shown, superimposed with low-energy conformations of loop 2 analogues L2-12, L2-10, L2-8 and L2-6, each of which is constrained by a disulphide bridge (indicated by arrows).

Figure 3 shows the concentration-response curves of monomeric cyclic loop 2 analogues in competition with BDNF. The monomeric cyclic loop 2 analogues L2-12 (closed triangles), L2-12a (open triangles), L2-10 (open squares), L2-8 (closed diamonds) and L2-6 (open diamonds) and the monomeric linear peptide L2-12b (closed squares) were assayed in competition with BDNF (4×10^{-11} M) in cultures of E8 to E10 chick sensory neurons. Surviving neurons were counted after 48 hrs in culture, and these counts were then expr ssed as a perc ntage of originally-plated viable neurons and normalised such that survival in cultures

containing BDNF alone (P; positive control; closed circle) was set to 100% and survival in cultures with neither BDNF nor loop 2 analogue (N; negative control; open circle) to 0%. Data are expressed as the mean ± SEM from at least 8 observations (n=8) from 4 independent experiments.

Figure 4 shows the concentration-response curves of monomeric cyclic peptide L2-12, alone and in competition with NGF. Surviving neurons were counted after 48 hrs in culture, and these counts expressed as a percentage of originally-plated viable neurons and normalised such that survival in cultures containing NGF alone (P; positive control; closed circle) was set to 100% and survival in cultures with neither NGF nor L2-12 (N; negative control; open circle) to 0%. When assayed in the absence of NGF, L2-12 (closed triangles) produced no significant effect on neuronal survival when compared to survival in negative control cultures ($p > 0.05$, ANOVA; n=6). L2-12 (open triangles) assayed in competition with NGF (4×10^{-11} M) produced no significant effect on NGF-mediated survival ($p > 0.05$, ANOVA; n=6). Data are expressed as the mean ± SEM.

Figure 5 shows the effect of a monomeric cyclic loop 2 analogue (L2-12a) on the concentration-response curve of BDNF. L2-12a (1×10^{-7} M) was assayed in competition with BDNF (1.8×10^{-13} to 1.8×10^{-10} M, 0.51 og increments) in cultures of E8-E10 sensory neurons. Surviving neurons were counted after 48 hrs in culture; these counts were expressed as a percentage of the number of originally plated viable neurons, and logistic sigmoidal curves fitted to the data. Compared to the BDNF control curve (closed circles), the BDNF curve in the presence of L2-12a (open circles) shows a significant depression in maximum (40%; ** $p < 0.005$, Student's unpaired two-tailed t-test) and a 1.6 fold shift in pEC_{50}, although the latter is insignificant ($p > 0.05$, Student's unpaired two-tailed t-test). N (closed square) refers to negative control cultures in the absence of both BDNF and L2-12a. Data are expressed as the mean ± SEM of 8 observations (n=8) from 4

independent experiments.

Figure 6 illustrate the maximal inhibition of BDNF-mediated survival of cultured sensory neurons by monomeric cyclic loop 2 analogues systematically substituted with alanine. Loop 2 analogues were assayed in competition with BDNF (4×10^{-11} M) in cultures of E8-E10 sensory neurons. Surviving neurons were counted after 48 hrs in culture, and these counts were expressed as a percentage of originally-plated viable neurons then normalised such that survival of cultures containing BDNF was set as 100%, while that for cultures with neither BDNF nor loop 2 analogue was set to 0%. Maximal inhibition of BDNF-mediated survival was calculated by subtracting the lowest value for BDNF-mediated survival from that of BDNF alone (100%). Note that alanine substitution in the L2-12 sequence can affect the ability of these peptides to modulate BDNF-mediated survival. Significant reduction in inhibitory activity, compared to L2-12 (closed bar), was observed when Ala was substituted for Val^3, Val^5, Ser^6 (***$p < 0.001$; ANOVA Bonferroni multiple comparisons test n=12), Lys^{11} (*$p < 0.05$; n=12) and Gln^{12} (**$p < 0.01$; n=10). No data (ND) were obtained for L2-12P4ΔA. Data are expressed as the mean ± SEM.

Figure 7A shows a schematic view of the two loop two regions in the model of the three-dimensional structure of the BDNF dimer, showing the interatomic distances (Å) between α-carbon atoms of selected residues.

Figure 7B shows a schematic view of the disulphide bridge of the cysteine residue, showing the average interatomic distance and 90% confidence interval (90% CI) of α-carbon atoms, determined by conformational analysis.

Figure 8 shows a graph of the survival of sensory neurons in the presence of the bicyclic dimeric peptides $(L2\text{-}8P2C)_2$, $(L2\text{-}8V3C)_2$ and (L2-8S4C)2. Neurons were prepared from chick dorsal root ganglia from embryonic chicks (E8-E10), and surviving n urons counted after 48 hrs

in culture. Data are presented as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. Highly significant differences in neuronal survival in the presence of $(L2\text{-}8P2C)_2$, and $(L2\text{-}8S4C)_2$ were observed compared to survival in negative controls (ANOVA, *** $p<0.001$, Bonferroni multiple comparisons test).

Figure 9 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic peptides L2-8P2C(Acm) and L2-8S4C(Acm). Neurons were prepared from dorsal root ganglia from embryonic chicks (E8-E10), and surviving neurons counted after 48 hours in culture. Data are presented as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. Data were obtained from at least two independent experiments.

Figure 10 shows a graph of the survival of sensory neurons in the presence of the amide-linked dimeric bicyclic peptide $(L2\text{-}8\&E+K)_2$. Neurons were prepared from chick dorsal root ganglia obtained from embryonic chicks (E8-E10), and surviving neurons counted after 48 hours in culture. Data are expressed as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. A highly significant difference in neuronal survival in the presence of $(L2\text{-}8\&E+K)_2$ was observed compared to survival in negative controls (ANOVA, $p<0.001$, Bonferroni multiple comparisons test).

Figure 11 shows a graph of the survival of sensory neurons in the presence of the dimeric tricyclic loop 2 analogue $(L2\text{-}8S4C\&E+K)_2$. Neurons were prepared from chick dorsal root ganglia obtained from embryonic chicks (E8-E10), and surviving neurons count d after 48 hrs in culture. Data are expressed as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same

period in culture. Survival in negative control cultures was set to 0%. A highly significant difference in neuronal survival in the presence of $(L2\text{-}8S4C\&E+K)_2$ was observed compared to survival in negative controls (ANOVA, $p<0.001$, Bonferroni multiple comparisons test).

Figure 12 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4-derived L4-3pA(II) (closed circles). Neurons were prepared from dorsal root ganglia from embryonic chicks (E8-E10) and surviving neurons counted after 48 hrs in culture. B: positive control (BDNF 1 ng/ml); N: negative control (no peptide). *** Significantly different to negative control (ANOVA, $p< 0.001$, Bonferroni multiple comparisons test, n = 12).

Figure 13 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4-derived peptides L4-3pA(I), L4-3pA(II) and L4-3Hx, and their linear homologues L4-3pAa and L4-3Hxa. All peptides were added at a concentration of 10^{-6} M. BDNF was added at 1 ng/ml. Neg: shows the survival in negative control cultures containing neither BDNF nor peptide. *** Significantly different to negative control (ANOVA, $p< 0.001$, Bonferroni multiple comparisons test, n = 12).

Figure 14 shows a graph of the effect of the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses) and L4-3Hxa (asterisks) on the neuronal survival effect mediated by BDNF (1 ng/ml). Over the concentration range tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on BDNF mediated neuronal survival.

Figure 15 shows a graph of the effect of the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses) and L4-3Hxa (asterisks) on th neuronal survival effect mediated by NGF (1 ng/ml). Over the concentration rang

tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on NGF mediated neuronal survival.

Figure 16 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4 peptides, L4-3Ap(I), L4-3Ap(II), L4-3AP(I) and L4-3AP(II). All peptides were added at a concentration of 10^{-6} M. BDNF was added at a concentration of 1 ng/ml. Neg shows the survival of control cultures containing neither BDNF nor peptide. None of the peptides exhibited an effect on neuronal survival that was significantly different to that seen in negative control cultures.

Figure 17 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4 peptides L4-3K3ApA and L4-3K4ApA. The peptides were added at a concentration of 10^{-6} M. BDNF was added at a concentration of 1 ng/ml. Neg shows the survival of control cultures containing neither BDNF nor peptide. Neither of the peptides exhibited an effect on neuronal survival that was significantly different to that seen in negative control cultures.

Figure 18 shows the solution structure of peptide L4-3pA(II) derived by nuclear magnetic resonance (NMR) spectroscopy. Depicted is an overlay of the twenty conformations of peptide L4-3pA(II) with the lowest target function in the software package DYANA, following 10,000 steps of simulated annealing followed by 2,000 steps on minimisation using the NMR-derived distance and dihedral angle constraints. Residues are labelled and numbered.

Figure 19 shows the effects of peptide L4-3pA(II) of the invention on neuronal loss following peripheral nerve lesion. This was accomplished by comparing the number of sensory neurones in the C8 dorsal root ganglia and motor neurons in the central region of the spinal cord in the lesioned side versus that in the intact contralateral side five days following nerve lesion, and administration of the peptide to the distal nerve stump.

Detailed Description of the Invention

The invention will now be described by way of reference only to the non-limiting examples, and to the Figures.

Abbreviations

BDNF	brain-derived neurotrophic factor
E	embryonic day
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
TFA	trifluoroacetic acid
NMR	nuclear magnetic resonance spectroscopy.

Materials

Mouse recombinant BDNF was a kind gift from Dr R Kolbeck and Professor Y-A Barde (Max-Planck-Institute for Psychiatry, Martinsried, Federal Republic of Germany). NGF, purified from male mouse submaxillary gland was purchased from Boehringer-Mannheim (Mannheim, Federal Republic of Germany). Fertilised chicken eggs were obtained from Research Poultry Farms (Research, Victoria, Australia), trypsin from Worthington (Freehold, NJ, U.S.A.), L-15 from GIBCO BRL (Grand Island, NY, U.S.A.), horse serum from CSL (Parkville, Victoria, Australia), Nunclon 10 cm tissue culture dishes from Nalge Nunc International (Roskilde, Denmark), Falcon Multiwell 48-well tissue culture plates from Becton Dickinson (Franklin Lakes, NJ, U.S.A.) and mouse laminin, isolated from Englebreth-Holm-Swarm tumour cells, from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Fmoc-amino acids and Wang resin were purchased from Auspep (Parkville, Victoria, Australia), PR-500 resin from Calbiochem-NovaBiochem (Alexandria, New South Wales, Australia) and Econosil irregular packed HPLC columns from Alltech Associates (Baulkham Hills, New South Wales, Australia). Other reagents were purchased from Sigma (Castle Hill, New

South Wales, Australia).

Example 1 Homology Modelling of BDNF

A model of the three-dimensional structure of murine BDNF was obtained by protein homology modelling techniques from murine NGF. This was performed by the Swiss-Model automated protein homology server running at the Glaxo Institute for Molecular Biology in Geneva, Switzerland, accessed via the Internet (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html, Peitsch, 1995). Briefly, a three-dimensional model of the target sequence is produced in the following manner: Swiss-Model searches the Brookhaven Protein Data Bank for the sequences of homologous proteins of known three-dimensional structure. Once a template sequence is found, Swiss-Model produces a structural framework for the target sequence, using a combination of sequence alignment tools and three-dimensional superimposition. Homologous regions of the template protein form the structural backbone of the target sequence, while non-conserved regions are built using the three-dimensional structures of related sequences in the Brookhaven Protein Data Bank. Side chains not present on the template protein are inserted, and all side chains are corrected using a library of allowed rotamers. The model is then optimised by energy minimisation using the CHARMm force-field.

The co-ordinates of the BDNF monomer were downloaded and the BDNF dimer constructed by superimposing two monomers over the co-ordinates of selected conserved α-carbon atoms in the NGF dimer, using the PC-based molecular modelling software Hyperchem version 4.0 (Hypercube, Ontario, Canada).

As expected, the model of the three-dimensional structure of BDNF obtained by homology modelling possessed an overall fold very similar to that of NGF, as shown in Figure 2. The validity of this structure was further confirmed by its similarity to the crystal structure of a

BDNF/NT-3 heterodimer (Robinson *et al*, 1995), whose co-ordinates were released some time after this work commenced.

Example 2 Molecular Modelling of Monomeric Cyclic Loop 2 Analogues

The molecular modelling of peptide analogues was carried out using Hyperchem, as follows: After visual inspection of the model obtained by homology modelling, loop 2 was defined and excised from the three-dimensional structure of BDNF, and various means of constraining peptides to the native loop 2 conformation were investigated. Each constraint was built between the terminal residues of the peptide, and these residues geometrically optimised using the Polak-Ribiere algorithm and MM+ force-field to a local low-energy conformation. These modelled peptides were assessed for their ability to mimic the native conformation by measuring the root mean square deviation of the peptide backbone to that of the native loop following least squares superimposition.

From this model of BDNF the second β-hairpin loop (loop 2) was defined as Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}, where the amino acid numbering is the same as in mature BDNF. Peptide analogues of this loop were modelled to investigate

(i) what type of constraint would be most appropriate to allow the peptides to mimic loop 2 in its native loop conformation, and

(ii) where in the sequence this constraint would be best positioned.

As a result of these studies four peptides, L2-12, L2-10, L2-8 and L2-6, each constrained by a disulphide bridge between terminal cysteine residues, were chosen for synthesis and biological examination. The sites from which these peptides are derived are illustrated in Figure 2.

Example 3 Synthesis of Monomeric Cyclic

Loop 2 Peptides

Linear peptides in the free acid form were assembled manually from Fmoc-amino acids on Wang resin using batch-type solid phase methods (Fields and Noble, 1990). Both coupling and deprotection reactions were assessed with the trinitrobenzenesulphonic acid test (Thompson *et al*, 1995). The linear peptide amide L2-12a was synthesised using continuous flow methods on PR-500 resin; coupling and deprotection steps were monitored spectrophotometrically. Cleavage of peptides from the resin and side chain removal was accomplished with trifluoroacetic acid (TFA)/ethanedithiol/H_2O (18:1:1).

Crude peptide products were analysed and purified by reversed phase HPLC over Econosil C-18 irregular packed columns. Gradients were tailored to individual runs, using combinations of solution A (0.1% TFA in H_2O) and solution B (0.1% TFA in 70% acetonitrile, 30% H_2O).

Purified peptide products were cyclised by oxidising terminal cysteine residues to the disulphide in the presence of 10% dimethylsulphoxide in 0.1 M NH_4HCO_3 solution at pH 8.0 (Tam *et al*, 1991). Reactions were monitored and the cyclised products purified by high performance liquid chromatography. The purity of the peptides was further assessed by capillary zone electrophoresis (Applied Biosystems 270A). The identity of each peptide was confirmed by mass spectrometry using either electrospray (Micromass platform II with electrospray source), or fast atom bombardment (Jeol JMS-Dx 300) techniques.

All the peptide analogues were synthesised with free amino and carboxyl termini, except L2-12a, which had acetylated amino and amidated carboxyl termini. The linear peptide L2-12b was synthesised without terminal Cys residues, to ensure that it remained in a linear form during biological assays. The peptides L2-12E1ΔA to L2-12Q12ΔA contain alanine substitution at the indicated positions in the L2-12 sequence.

The peptide analogues synthesised are listed in Table 1.

Table 1

Monomeric Cyclic Loop 2 Analogue Sequences

Code	Peptide Analogue Sequence	SEQ ID NO.
L2-12	C-E-K-V-P-V-S-K-G-Q-L-K-Q-C	SEQ ID NO.1
L2-12a	Ac-C-E-K-V-P-V-S-K-G-Q-L-K-Q-C-NH_2	SEQ ID NO.2
L2-12b	E-K-V-P-V-S-K-G-Q-L-K-Q	SEQ ID NO.3
L2-10	C-K-V-P-V-S-K-G-Q-L-K-C	SEQ ID NO.4
L2-8	C-V-P-V-S-K-G-Q-L-C	SEQ ID NO.5
L2-6	C-P-V-S-K-G-Q-C	SEQ ID NO.6
L2-12E1ΔA	C-A-K-V-P-V-S-K-G-Q-L-K-Q-C	SEQ ID NO.7
L2-12K2ΔA	C-E-A-V-P-V-S-K-G-Q-L-K-Q-C	SEQ ID NO.8
L2-12V3ΔA	C-E-K-A-P-V-S-K-G-Q-L-K-Q-C	SEQ ID NO.9
L2-12P4ΔA[a]	C-E-K-V-A-V-S-K-G-Q-L-K-Q-C	SEQ ID NO.10
L2-12V5ΔA	C-E-K-V-P-A-S-K-G-Q-L-K-Q-C	SEQ ID NO.11
L2-12S6ΔA	C-E-K-V-P-V-A-K-G-Q-L-K-Q-C	SEQ ID NO.12
L2-12K7ΔA	C-E-K-V-P-V-S-A-G-Q-L-K-Q-C	SEQ ID NO.13
L2-12G8ΔA	C-E-K-V-P-V-S-K-A-Q-L-K-Q-C	SEQ ID NO.14
L2-12Q9ΔA	C-E-K-V-P-V-S-K-G-A-L-K-Q-C	SEQ ID NO.15
L2-12L10ΔA	C-E-K-V-P-V-S-K-G-Q-A-K-Q-C	SEQ ID NO.16
L2-12K11ΔA	C-E-K-V-P-V-S-K-G-Q-L-A-Q-C	SEQ ID NO.17
L2-12Q12ΔA	C-E-K-V-P-V-S-K-G-Q-L-K-A-C	SEQ ID NO.18

Amino acids are represented by their one letter code, reading left to right from amino to carboxyl termini. The analogue code, for example L2-12K9ΔA, refers to 12 residues from the native loop 2 sequence of BDNF with lysine at position 9 substituted with alanine. Cysteine residues not found in the native BDNF sequence were incorporated to form disulphide bridges, which are represented by lines between side chains.

[a]peptide L2-12P4ΔA not synthesised.

Exampl 4 Inhibition of BDNF-Mediated Sensory Neuron Survival by Monomeric Cyclic Loop 2 Peptides

The biological activity of the monomeric cyclic loop 2 peptide analogues was assayed in primary cultures of sensory neurons prepared from embryonic chick dorsal root ganglia, essentially as described by Barde *et al* (1980). The survival of specific sub-populations of these neurons in culture is supported by neurotrophins acting through the appropriate member of the trk receptor family (Lindsay, 1996). Briefly, 80 dorsal root ganglia were dissected from four embryonic day 8-10 chicks (E8-E10), treated with 0.1% trypsin for 20 min at 37°C, washed twice with 2 ml medium (L-15 (CO_2), 5% horse serum, 60 µg/ml streptomycin and 100 µg/ml penicillin) and gently triturated. Non-neuronal cells were removed by pre-plating the neuronal suspension on a 10 cm tissue culture dish for 3 h at 37°C, 5% CO_2. Prior to plating of neurons, 48-well tissue culture plates were coated with poly-DL-ornithine (150 µl of 1 mg/ml in 0.15 M sodium borate buffer pH 8.3) overnight at 4°C and then with laminin (125 µl of 7.5 µl/ml in L-15 (CO_2)) for 4 hrs at 37°C, 5% CO_2. Immediately after removal of laminin solution, 200 µl of suspension, and where appropriate, samples of test compounds (2 µl) were added to each well. After 1 h neurotrophic factors were added (either 2 or 6 µl), and viable neuron numbers were determined manually by counting 40 standard fields at 200x magnification.

After 48 hrs incubation, phase-bright healthy neurons with neurites at least twice the length of the cell soma were counted in 20-30 fields at 200x magnification, and counts expressed as a percentage of the original number of viable neurons plated (% neuronal survival). Percentage neuronal survival data was normalised, such that neuronal survival in the presence of neurotrophin (4 x 10^{-11} M; positive control) was set to 100%, while survival in the absence of both neurotrophin and monomeric cyclic loop 2 peptide analogue (negative control) was s t to 0%. Values were expressed as mean ± SEM. Data from different

experiments were analysed for lack of significant variation using a parametric one-way analysis of variance (ANOVA) before being grouped. Statistics were performed using Instat version 2.04a (GraphPad, San Diego, CA, U.S.A.). Prism software (GraphPad, San Diego, CA, U.S.A.) was used to fit sigmoidal curves to the data.

To investigate the ability of the loop 2 analogues L2-12, L2-12a, L2-10, L2-8 and L2-6 to modulate BDNF-mediated survival, the peptides were assayed from 1 x 10^{-11} to 1 x 10^{-4} M in competition with BDNF at 4 x 10^{-11} M, a concentration which produces near maximal survival. The results are summarised in Figure 3. All five peptides showed a similar pattern of concentration-dependent inhibition of BDNF-mediated survival, causing an increase in inhibition from 1 x 10^{-11} to a maximum at approximately 1 x 10^{-6} M; above this concentration inhibition either reached a plateau (L2-10), or diminished (L2-12, L2-12a, L2-8 and L2-6), giving the concentration-response curve an inverted bell-shape. However, the maximal level of inhibition produced by these peptides varied: L2-8 showed the greatest maximum (50% ±5), followed by L2-12a (44% ±4), L2-10 (41% ±2), L2-12 (40% ±3) and L2-6 (27% ±6). The maximal inhibition and pIC_{50} values, the latter obtained from logistic sigmoidal curves fit to the data, are summarised in Table 2.

Table 2

Summary of Data for L2-12, L2-12a, L2-10, L2-8 and L2-6 in Competition with BDNF in Cultures of E8-E10 Sensory Neurons

Loop 2 analogue	Maximal inhibition	pIC_{50}
L2-12	40% ± 3	9.93 ± 0.15
L2-12a	44% ± 4	9.38 ± 0.30
L2-10	41% ± 2	8.16 ± 0.26
L2-8	50% ± 5	9.54 ± 0.16
L2-6	27% ± 6	10.53 ± 0.16

Maximal inhibition refers to the greatest % reduction in normalised % BDNF-mediated neuronal survival. pIC_{50} values were calculated from logistic sigmoidal curves fitted to the data given in Figure 3.

In contrast to the results obtained with the monomeric cyclic peptides, the monomeric linear peptide L2-12b did not show significant inhibition of BDNF-mediated survival over the concentration range tested (1×10^{-11} to 1×10^{-4} M; Figure 3).

Example 5 Specificity of Inhibition of Neuronal Survival Activity by Monomeric Cyclic Loop 2 Analogues

To determine the specificity of the peptides in inhibiting BDNF-mediated survival, monomeric cyclic peptide L2-12 (1×10^{-11} to 1×10^{-4} M) was assayed in competition with NGF (4×10^{-11} M), using the assay described in Example 4. As shown in Figure 4, at the concentrations tested peptide L2-12 did not significantly inhibit NGF-mediated survival. These data suggest that the inhibition of neuronal survival seen in Example 4 is specific for BDNF.

Example 6 Lack of Intrinsic Neuronal Survival Activity or Toxic Effects of Monomeric Cyclic Loop 2 Analogues

When added to cultures alone, *i.e.* in the absence of neurotrophin, the monomeric cyclic peptide L2-12 neither intrinsically promoted neuronal survival nor exhibited non-specific toxic effects on neurons at the concentrations tested (1×10^{-11} to 1×10^{-4} M), giving neuronal survival of around 5%, *i.e.* similar to that of negative controls, as shown in Figure 4.

This lack of intrinsic neuronal survival promoting activity of the monomeric cyclic loop 2 peptide L2-12 was expected. L2-12 and the other monomeric cyclic loop 2 peptides are monomeric, they are unlikely to dimerise trkB, and the dimerization is crucial for trk-mediated signalling (Jing *et al*, 1992).

Example 7 Effect of Monomeric Cyclic Peptides on the Concentration-Response Curve for BDNF

To examine the effect of inhibitory peptides on the concentration-response curve of BDNF, monomeric cyclic L2-12a (1×10^{-7} M) was added in competition with BDNF (1.8×10^{-13} to 1.8×10^{-10} M in 0.5 log increments). The results are shown in Figure 5. At this concentration, peptide L2-12a caused a significant depression (40%) of the maximal % neuronal survival response of BDNF from 41% to 24%, which is consistent with the maximal inhibition of BDNF-mediated survival exhibited by L2-12a. The peptide also caused a small rightward shift of the BDNF concentration-response curve (pEC_{50} of BDNF alone: 11.2 ±0.2; BDNF + L2-12a: 10.9 ±0.3), although this was not statistically significant.

Example 8 Identification of Amino Acids Important for the Inhibitory Effect of Monomeric Cyclic Loop 2 Peptides

The contribution of individual residues within the monomeric cyclic L2-12 sequence towards BDNF-inhibitory activity was examined by conducting an alanine scan (Ala scan), and testing the resulting monomeric cyclic peptides from 1×10^{-11} to 1×10^{-4} M in log increments for their ability to modulate BDNF-mediated survival at 4×10^{-11} M. The sequences of the alanine substituted peptides are shown in Table 1 above, and the results are shown in Figure 6.

A significant difference in maximal inhibition compared to that produced by L2-12 (40% ±3) was seen when Ala was substituted for Val^{3} (Val^{42} in native BDNF sequence; 0% ±9), $Ser^{6(45)}$ (2% ±7), $Lys^{11(50)}$ (9% ±4) and $Gln^{12(51)}$ (5% ±5), suggesting that these residues are important for BDNF-inhibitory activity. Substitution of Ala for $Val^{5(44)}$ yielded a peptide which gave a slight, though insignificant, potentiation of BDNF-mediated survival (-7% ±9). However, the peptide did not show intrinsic survival promoting activity in the abs nce of BDNF (data not shown). No significant change in maximal inhibition was observed

when Ala was substituted for $Glu^{1(40)}$ (39% ±13), $Lys^{2(41)}$ (26% ±7), $Lys^{7(46)}$ (33% ±8), $Gly^{8(47)}$ (46% ±13), $Gln^{9(48)}$ (32% ±6) and $Leu^{10(49)}$ (33% ±6).

Example 9 Molecular Design of Disulphide-linked Dimeric Bicyclic Loop 2 Analogues

The two loop 2 regions of BDNF are juxtaposed in the three-dimensional model of the dimer (Figure 1), which allows design of small dimeric peptides that mimic this spatial arrangement. On the basis of observations made in the highly analogous NGF-trkA receptor system, we predicted that small dimeric loop 2 analogues could act as agonists if they could facilitate dimerization of trkB. It has been shown that divalent antibodies to trkA can cause the homodimerisation of this receptor, leading to signal transduction and NGF-like biological activity *in vitro* (Clary *et al*, 1994). Moreover, a small peptide mimetic of erythropoietin, produced by a recombinant library technique, possesses full erythropoietin-like biological activity as a result of self-association to form a dimer which dimerises the erythropoietin receptor (Wrighton *et al*, 1996). Examination of the X-ray crystal structure of the peptide-erythropoietin receptor complex (Livnah *et al*, 1996) reveals that the structure of the bound dimeric peptide bears a striking resemblance to the loop 2 regions of BDNF in our three-dimensional model.

The most effective of the monomeric, cyclic, disulphide-linked loop 2 peptides which were shown in Examples 4, 5, 7 and 8 were able to inhibit BDNF neuronal survival activity, peptide L2-8, was chosen as the basis for the design of dimeric peptides. This peptide consists of 8 amino acid residues of BDNF plus the two terminal cysteine residues oxidised to cyclic disulphide, *i.e.* a total of 10 residues.

Examination of the model of the three-dimensional structure of BDNF revealed three amino acid positions in which the two loop 2 regions in BDNF are in close

proximity, thus presenting an opportunity to create dimeric analogues of peptide L2-8. The positions, corresponding to Pro^2, Val^3 and Ser^4 of peptide L2-8, are characterised by Cα-to-Cα distances of 11.3Å, 5.4Å and 6.2Å, respectively, as shown in Figure 7A. Conformational analysis of a cysteine residue (*i.e.* two disulphide-linked cysteine residues) by computational chemical methods revealed that the mean Cα-to-Cα distance of this residue was 5.4Å (90% CI: 5.22-5.44Å), as shown in Figure 7B. These data suggested to us that a cysteine residue could comfortably be incorporated into peptide L2-8 at positions Val^3 and Ser^4, to give dimeric, disulphide-linked peptides that might mimic the spatial arrangement of the loop 2 regions in native BDNF, but fitted considerably less well in place of Pro^2.

However, as shown in Example 8, examination of a series of peptides based on a 14 amino acid monomeric loop 2 peptide inhibitor of BDNF action, in which amino acids were systematically replaced with Ala, showed that residues equivalent to Val^3 and Ser^4 in peptide L2-8 were required for BDNF inhibitory activity, and therefore were presumably involved in binding to BDNF receptors. No data were available concerning an Ala replacement at a position equivalent to Pro^2.

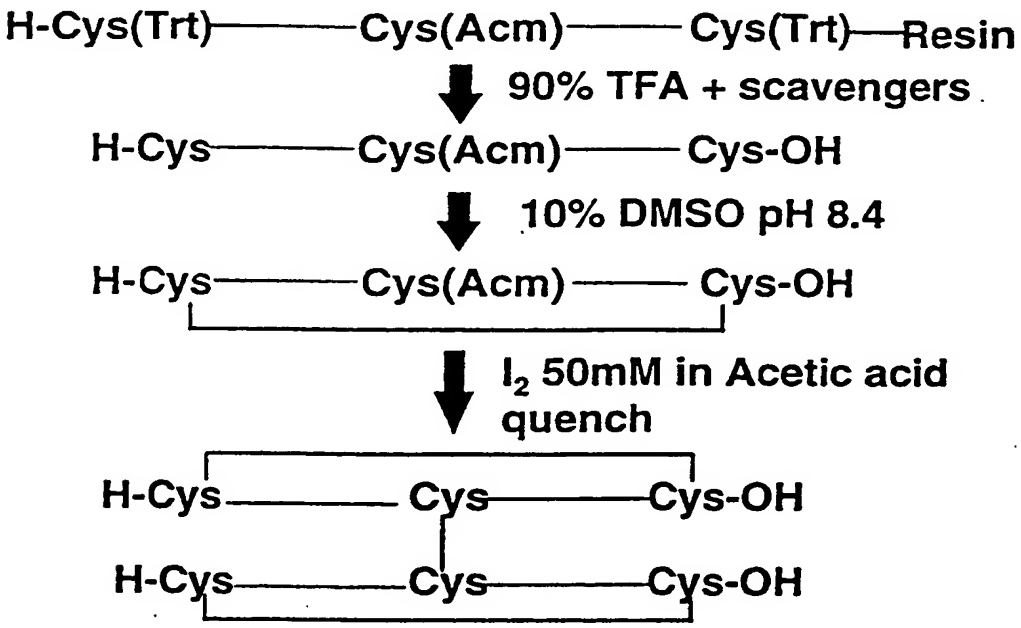
On the basis of the structural data alone, we chose to synthesise the, disulphide-linked dimeric bicyclic peptides $(L2\text{-}8V3C)_2$ and $(L2\text{-}8S4C)_2$, incorporating a cysteine bridge in place of Val^3 and Ser^4, respectively. The disulphide-linked dimeric bicyclic peptide $(L2\text{-}8P2C)_2$, in which the cysteine linkage was incorporated in place of Pro^2, was also synthesised, as definitive information on the role of Pro^2 was not available, even though our structural data suggested that cysteine would not provide the optimal means of dimerization at this point.

Example 10 Synthesis of Disulphide-Linked Dimeric Bicyclic Loop 2 Analogues

The disulphide-linked dimeric bicyclic peptides $(L2\text{-}8P2C)_2$, $(L2\text{-}8V3C)_2$ and $(L2\text{-}8S4C)_2$ were synthesised by standard solid phase synthesis techniques using Fmoc amino acids, as described in Example 3, and using a mixed Cys protection strategy (Cys(Trt) and Cys(Acm)). The general method is illustrated in Scheme 1.

Scheme 1

Synthesis of disulphide-linked dimeric bicyclic peptide analogues of loop 2 of BDNF



Peptides and intermediates were purified by reverse-phase high performance liquid chromatography, and characterised by electrospray mass spectrometry. The structures of the compounds synthesised are shown in Table 3.

Table 3

Structures of Disulphid -linked Dimeric Bicyclic Loop 2 Analogues and Their Monomeric Cyclic Precursors

L2-8	C-V-P-V-S-K-G-Q-L-C	
$(L2\text{-}8P2C)_2$	C-V-C-V-S-K-G-Q-L-C C-V-C-V-S-K-G-Q-L-C	SEQ ID NO.19
$(L2\text{-}8V3C)_2$	C-V-P-C-S-K-G-Q-L-C C-V-P-C-S-K-G-Q-L-C	SEQ ID NO.20
$(L2\text{-}8S4C)_2$	C-V-P-V-C-K-G-Q-L-C C-V-P-V-C-K-G-Q-L-C	SEQ ID NO.21
L28P2C(Acm)	C-V-C(Acm)-V-S-K-G-Q-L-C	SEQ ID NO.22
L28V3C(Acm)	C-V-P-C(Acm)-S-K-G-Q-L-C	SEQ ID NO.23
L28S4C(Acm)	C-V-P-V-C(Acm)-K-G-Q-L-C	SEQ ID NO.24

Example 11 Intrinsic Neuronal Survival Activity of Disulphide-Linked Dimeric Bicyclic Loop 2 Analogues

The disulphide-linked dimeric bicyclic loop 2 peptides were analysed for their ability to promote the survival of sensory neurons in cultures prepared from dorsal root ganglia obtained from embryonic day 8-10 chicks, as described in Example 4. Peptides $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ each displayed concentration-dependent neuronal survival activity, maximally promoting the survival of 28% and 30% of the neurons that would be supported by BDNF itself. These results are shown in Figure 8.

The activity of these two disulphide-linked dimeric bicyclic loop 2 peptides was surprising in view of the number of the modifications. In peptide $(L2\text{-}8S4C)_2$, the serine residue (Ser^4) shown to be important for the inhibitory action on BDNF-mediated neuronal survival of the monomeric cyclic loop 2 peptides (see Example 8) was replaced by the disulphide-linked cysteine residue. In peptide $(L2\text{-}8P2C)_2$, the Cα-to-Cα distance of the cysteine residue is likely to be much shorter than the corresponding distance in our model of BDNF of the two proline residues which it replaces.

In contrast, peptide $(L2\text{-}8V3C)_2$ was inactive, as shown in Figure 8, despite the likelihood that it could best accommodate the cysteine residue, at least in terms of interatomic distance.

Example 12 Lack of Intrinsic Neuronal Survival Activity of Monomeric Precursors of Dimeric Bicyclic Loop 2 Analogues

To determine whether the dimeric nature of the disulphide-linked dimeric bicyclic loop 2 peptides was required for intrinsic neuronal survival activity, we assayed peptides L2-8P2C(Acm) and L2-8S4C(Acm), the monomeric cyclic precursors of peptid s $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ in which the Acm groups on the internal Cys were

intact, for their ability to promote sensory neuronal survival in culture. Unlike their dimeric counterparts, both monomeric cyclic peptides were inactive, as shown in Figure 9.

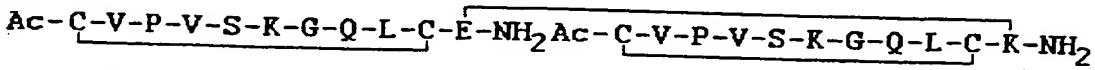
Example 13 Molecular Design of an Amide-Linked Dimeric Bicyclic Loop 2 Analogue

Because of the position of the cysteine residue in the disulphide-linked dimeric bicyclic loop 2 analogues described in Examples 9 to 11 in the region required for the inhibitory activity of the monomeric cyclic peptides, we further examined the BDNF model to see if other dimeric bicyclic peptides could be designed that did not involve the replacement of these possible receptor-binding residues. On the basis of Cα-to-Cα distance measurements. we reasoned that an amide-linked dimeric bicyclic peptide, $(L2\text{-}8\&E+K)_2$, could be created by joining together two analogues of the monomeric cyclic peptide L2-8 via an amide bond between an additional lysine and glutamate residue added to the C-terminus. An illustration of this is shown in Table 4.

Table 4

Structure of Amide-Linked Bicyclic Analogue of Loop 2 of BDNF

$(L2\text{-}8\&E+K)_2$



SEQ ID NO. 25

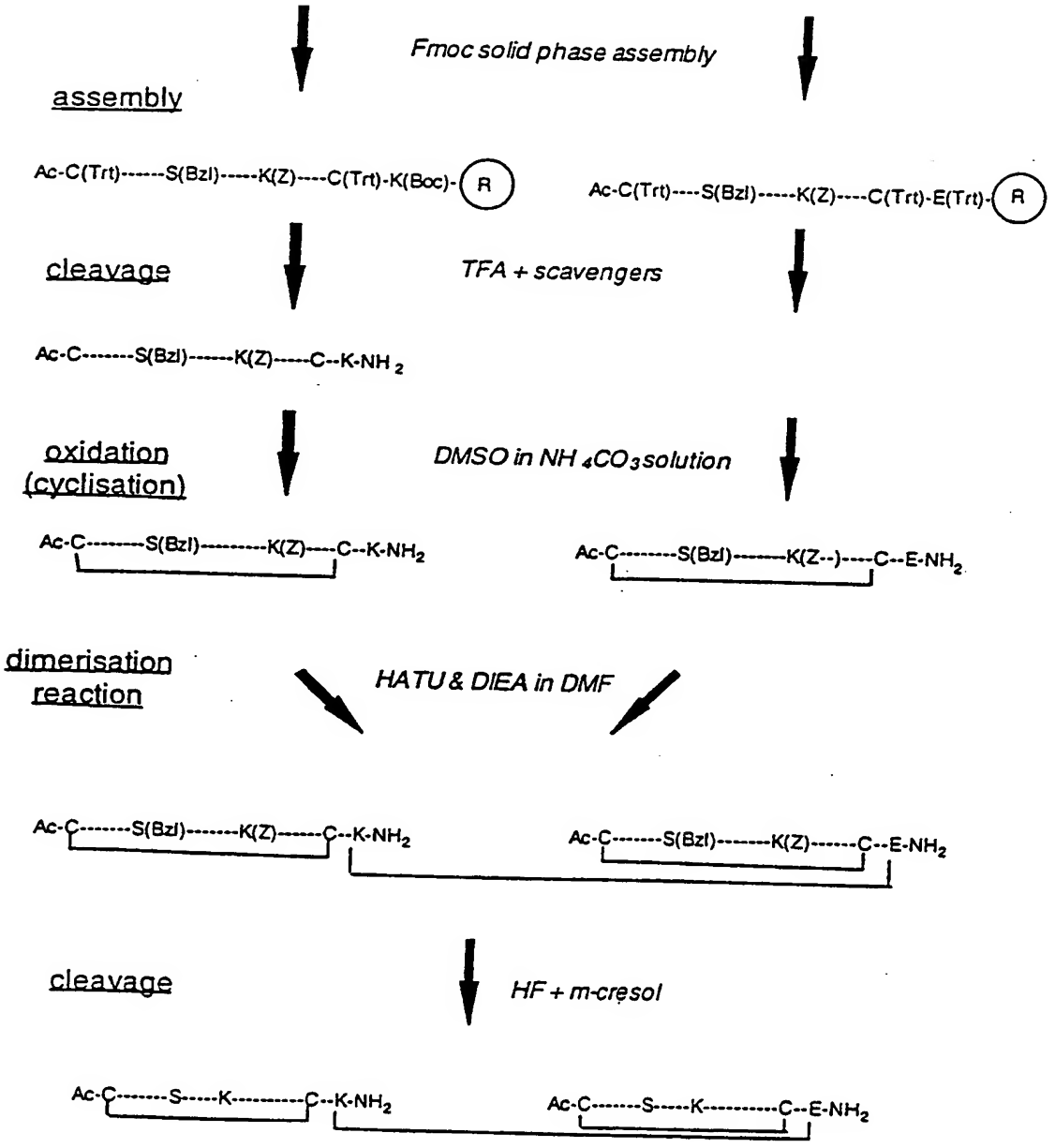
SEQ ID NO. 26

Example 14 Synthesis of an Amide-Linked Dimeric Bicyclic Loop 2 Analogue

The amide-linked dimeric bicyclic peptide (L2-8&E+K)$_2$ was prepared as shown in Scheme 2 by condensing two cyclic N-acetylated, C-amidated, partially-protected monomers synthesised by standard solid phase techniques on Rink amide MBHA resin as described in Example 3 and shown in Scheme 2.

Scheme 2

Synthesis of an amide-linked dimeric bicyclic loop 2 analogue



The remaining Ser and Lys protecting groups were removed by treatment of the partially protected bicyclic dimer with hydrogen fluoride/m-cresol (10:1) for one hour at 5°C. HF was removed by evaporation at room temperature. The desired peptide $(L2\text{-}8\&E+K)_2$ and intermediates were purified by HPLC and characterised by mass spectrometry.

Example 15 Intrinsic Neuronal Survival Activity of an Amide-Linked Dimeric Bicyclic Loop 2 Analogue

The amide-linked dimeric bicyclic loop 2 peptide $(L2\text{-}8\&E+K)_2$ was assayed in cultures of sensory neurons prepared from dorsal root ganglia obtained from embryonic chicks, as described in Example 4. Peptide displayed concentration dependent neuronal survival activity, supporting the survival of 28% of those neurons supported by BDNF (1ng/ml) with an EC_{50} in the order of 10^{-8} M. The results are shown in Figure 10.

This activity was similar both in maximal effect and potency to that observed with the disulphide-linked dimeric bicyclic loop 2 analogues $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ described in Example 11. Thus it appears that different chemical linkers incorporated in different positions within the dimeric bicyclic loop 2 analogues imparts neuronal survival promoting activity to these peptides.

Example 16 Molecular Design of a Dimeric Tricyclic Loop 2 Analogue

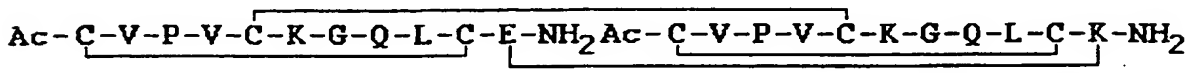
Although the dimeric bicyclic peptides described in Examples 9 through 11 and 13 through 15 represent a significant step in the discovery of small molecules which mimic the action of BDNF, they are nonetheless considerably less potent efficacious than the parent peptide. One reason for the reduced activity of the dimeric bicyclic peptides compared to BDNF could be their ability to rotate relatively freely about their dimerising constraint, be it a Cys-to-Cys disulphide or a Lys-to-Glu amide. To try and

create a molecule that might show either improved efficacy (as evidenced by an increase in the maximal percent neuronal survival), or increased potency, we reasoned that we would need to restrict the freedom of rotation about the dimerising constraint. To do this, we chose to combine, in one molecule, the two different dimerising constraints used in the disulphide-linked and the amide-linked dimeric bicyclic loop 2 analogues. We anticipated that the resultant dimeric tricyclic loop 2 analogue $(L2\text{-}8S4C\&E+K)_2$, by restricting the rotation of the two loop 2 moieties relative to one another would much better mimic the loop 2 orientation seen in the native protein, and therefore would show improved efficacy and potency. This is shown in Table 5.

Table 5

Structure of Dimeric Tricyclic Loop 2 Analogue

$(L2\text{-}8S4C\&E+K)_2$



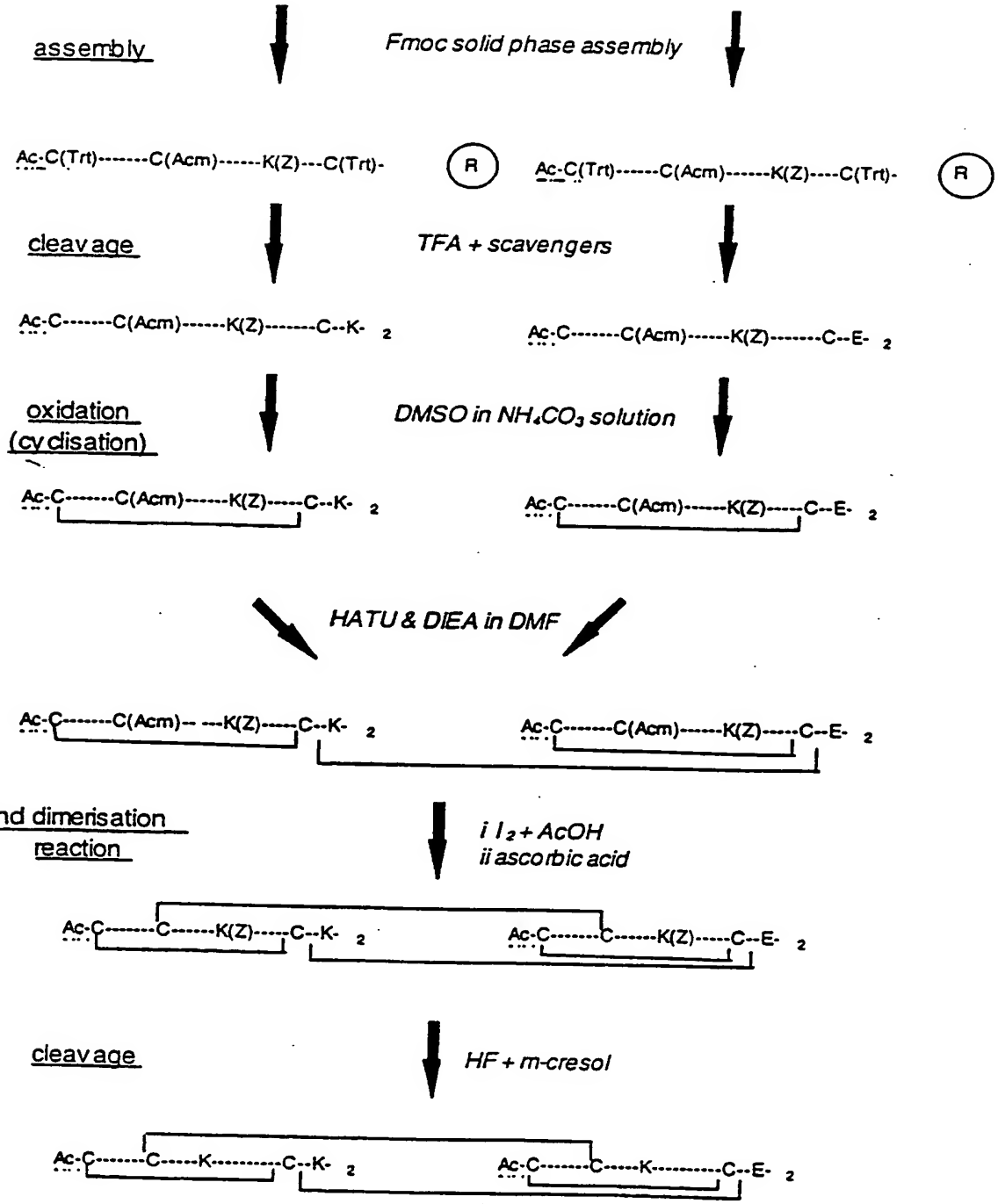
SEQ ID NO. 27 SEQ ID NO. 28

Example 17 Synthesis of a Dimeric Tricyclic Loop 2 Analogue

The dimeric tricyclic peptide (L2-8S4C&E+K)$_2$ was prepared as shown in Scheme 3 from two cyclic N-acetylated, C-amidated, partially-protected monomers synthesised by standard solid phase techniques on Rink amide MBHA resin as described in Example 14.

Scheme 3

Synthesis of a dimeric tricyclic loop 2 analogue



The monomers were initially condensed in the first dimerisation reaction via the free lysine and glutamate side chains. A second dimerisation reaction was carried by oxidising the internal cysteine residues, completing the tricycle. The remaining Lys protecting groups were removed by treating the partially protected tricyclic dimer with hydrogen fluoride/m-cresol (10:1) for one hour at 5°C. HF was removed by evaporation at room temperature. The desired peptide (L2-8S4C&E+K)$_2$ and intermediates were purified by HPLC and characterised by mass spectrometry.

Example 18 Intrinsic Neuronal Survival Activity of a Dimeric Tricyclic Loop 2 Analogue

The dimeric tricyclic loop 2 peptide (L2-8S4C&E+K)$_2$ was assayed in cultures of sensory neurons prepared from dorsal root ganglia obtained from embryonic chicks, as described in Example 4. Peptide displayed concentration dependent neuronal survival activity, supporting the survival of 35% of those neurons supported by BDNF (1ng/ml) with an EC_{50} in the order of 10^{-10} M. The results are shown in Figure 11.

The maximal neuronal survival promoting effect of the dimeric tricyclic loop 2 peptide (L2-8S4C&E+K)$_2$ is similar to that of the dimeric bicyclic loop 2 analogues. However peptide (L2-8S4C&E+K)$_2$ is approximately two orders of magnitude more potent than the dimeric bicyclic analogues. This activity is consistent with the hypothesis that the presence of two dimerising constraints (Cys-to-Cys disulphide and Lys-to-Glu amide) would create a molecule which much better mimics the spatial arrangement of the two loop 2 moieties than any of the dimeric bicyclic compounds, which contain only a single dimerising constraint.

Example 19 Molecular Design of Monomeric Cyclic Analogues of the P75 Binding Region of Loop 4 of BDNF

The three positively-charged residues thought to

be important for the binding of BDNF to the low affinity neurotrophin receptor p75 are contiguous (Lys^{95}-Lys^{96}-Arg^{97}) and are located on loop 4, as shown in Figure 1. This gave us the opportunity to propose small monomeric cyclic peptides that might mimic the conformation of this tripeptide sequence, using the computer-aided molecular design approach described in Example 2. On the basis of these studies we chose to synthesise two cyclic monomeric peptides: L4-3pA, a pentapeptide incorporating a relatively conformationally restricted DPro residue; and L4-3Hx, a tetrapeptide incorporating a conformationally flexible 6-aminohexanoyl residue. Both peptides were cyclised by condensing their amino-terminus with their carboxy-terminus (head-to-tail cyclisation).

Example 20 Synthesis of Monomeric Cyclic Analogues of the p75 Binding Region of Loop 4 of BDNF

The monomeric cyclic loop 4 peptides were synthesised from 9-fluorenylmethoxycarbonyl (Fmoc) amino acids, using standard solid phase synthesis protocols as described in Example 3. The linear side chain-protected peptides L4-3pAa and L4-3Hxa, suitable for head-to-tail cyclisation to give the monomeric cyclic peptides L4-3pA and L4-3Hx, respectively, were obtained by treating peptides synthesised on acid-labile 2-chlorotrityl derivatised resin (NovaBiochem, Australia) with acetic acid/trifluoroethanol/dichloromethane (1:1:8) for 30 minutes (Barlos *et al.*, 1991). The cyclic peptides were obtained by stirring the appropriate linear side chain-protected peptide (0.1 to 0.5 mg/ml) in dichloromethane in the presence of the standard peptide bond-formation reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorphosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and diisopropylamine (DIEA) (HBTU:HOBt:DIEA 1:1:1.5 equivalents relative to peptide). Treatment of the product of this reaction with TFA/scaveng rs yielded the desired fully-deprotected product. The corresponding linear

homologues were prepared by treating the appropriate side chain-protected linear peptide with TFA/scavengers, without prior cyclisation.

Cyclisation reactions were monitored and peptides purified by reverse phase HPLC on either analytical (4.6 mm internal diameter) or semi-preparative (22.5 mm) C18 columns, using linear acetonitrile gradients in 0.1% TFA solution at appropriate flow rates. Desired fractions were collected and lyophilised for characterisation by mass spectrometry.

Synthesis of peptide L4-3pA yielded two stereoisomers, L4-3pA(I) and L4-3pA(II), each with the desired molecular weight of 581 daltons. These isomers were purified by HPLC and were assayed separately for biological activity.

A list of the compounds synthesised is given in Table 6.

Table 6

Structures of Monomeric Cyclic Loop 4 Analogues and Their Linear Homologues

L4-3pA(I) and L4-3pA(II)	┌DPro-Ala-Lys-Lys-Arg┐	SEQ ID NO.29
L4-3Hx	┌Ahx-Lys-Lys-Arg┐	SEQ ID NO.30
L4-3pAa	H-DPro-Ala-Lys-Lys-Arg-OH	SEQ ID NO.31
L4-3Hxa	H-Ahx-Lys-Lys-Arg-OH	SEQ ID NO.32

All amino acid residues are given by their standard three letter codes, except Ahx: 6-amino hexanoyl.

Example 21 Intrinsic Neuronal Survival Activity of a Monomeric Cyclic Analogue of the p75 Binding Region of Loop 4 of BDNF

The monomeric cyclic analogues of the p75 binding region of loop 4, L4-3pA(I), L4-3pA(II), L4-3Hx and their linear homologues L4-3pAa and L4-3Hxa, were assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. As shown in Figure 10, the monomeric cyclic loop 4 peptide, L4-3pA(II) displayed concentration-dependent neuronal survival activity. This intrinsic neuronal survival activity of L4-3pA(II) was surprising; unlike the loop 2 peptides described in Example 11, it is neither dimeric nor bicyclic. Moreover, the activity was confined to L4-3pA(II). Neither its stereoisomer L4-3pA(I), the other monomeric cyclic loop 4 peptide L4-3Hx constrained by the more conformationally flexible aminohexanoyl residue, nor their linear counterparts displayed neuronal survival activity in this assay system, as shown in Figure 11.

Example 22 Lack of Inhibition of BDNF- and NGF-Mediated Sensory Neuron Survival by Monomeric Cyclic Loop 4 Analogues of the p75 Binding Region of Loop 4 of BDNF and Their Linear Homologues

The monomeric cyclic analogues of the p75 binding region of loop 4 L4-3pA(I), L4-3pA(II), L4-3Hx and their linear homologues L4-3pAa and L4-3Hxa, were assayed for their ability to modulate the neuronal survival effects of BDNF and NGF in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Unlike the monomeric cyclic loop 2 peptides, none of the monomeric cyclic loop 4 peptides or their linear homologues showed any significant inhibition of either BDNF- or NGF- mediated neuronal survival, as shown in Figures 12 and 13, respectively.

Example 23 Role of DPro in the Neuronal Survival Promoting Activity of Monomeric Cyclic Loop 4 Analogues of the p75 Binding Region of BDNF

Given the biological data obtained with L4-3pA(II) described in Example 21, we decided to investigate the role of the DPro residue in the neuronal survival activity of the monomeric cyclic loop 4 peptides. We chose to synthesise two compounds using the methods described in Example 20: L4-3Ap, in which the position of the DPro residue is swapped with the Ala residue; and L4-3AP, in which the configuration of the Pro residue is L rather than D. In a manner presumably analogous to that seen with L4-3pA, both peptides yielded two isomers of identical molecular weight: L4-3Ap(I), L4-3Ap(II), L4-3AP(I) and L4-3AP(II). The sequences of these four peptides is shown in Table 7.

Table 7

Structure of Further Monomeric Cyclic Loop 4 Analogues

L4-3Ap(I) and L4-3Ap(II)	⌐Ala-ᴅPro-Lys-Lys-Arg¬	SEQ ID NO.33
L4-3AP(I) and L4-3AP(II)	⌐Ala-Pro-Lys-Lys-Arg¬	SEQ ID NO.34

The monomeric cyclic peptides were assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Unlike peptide L4-3pA(II), neither L4-3Ap(I), L4-3Ap(II), L4-3AP(I) nor L4-3AP(II) displayed neuronal survival activity. These data, shown in Figure 16, suggest that both the position in the cyclic sequence and stereochemistry of the Pro residue are important for the neuronal survival activity displayed by peptide L4-3pA(II).

Example 24 Role of Lys Residues in the Neuronal Survival Promoting Activity of Monomeric Cyclic Loop 4 Analogues of the p75 Binding Region of BDNF

To investigate the importance of the two Lys residues to the neuronal survival activity of the monomeric cyclic loop 4 peptides, we chose to synthesise two analogues of peptide L4-3pA using the methods described in Example 20 in which a Lys residue is replaced by Ala: L4-3K3ApA and L4-3K4ApA. Unlike L4-3pA, both L4-3K3ApA and L4-3K4ApA yielded only single major products following synthesis and cyclisation. The sequences of these two peptides is shown in Table 8.

Table 8

Structure of Further Monomeric Cyclic Loop 4 Analogues Incorporating Ala for Lys

L4-3K3ApA	⌐DPro-Ala-Ala-Lys-Arg¬	SEQ ID NO.35
L4-3K4ApA	⌐DPro-Ala-Lys-Ala-Arg¬	SEQ ID NO.36

The monomeric cyclic peptides L4-3K3ApA and L4-3K4ApA wer assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Compared to L4-3pA(II), peptides L4-3K3ApA and L4-3K4ApA displayed only marginal neuronal survival activity. These data, shown in Figure 17, suggest that the two Lys residues of the cyclic monomeric peptide L4-3pA are required for neuronal survival activity.

Example 25 NMR Analysis of Monomeric Cyclic Analogue of the p75 Binding Region of Loop 4 of BDNF

The neuronal survival activity of the monomeric cyclic analogues of the p75 binding region of loop 4 of BDNF is confined almost exclusively to peptide L4-3pA(II). To examine a structural basis for this neuronal survival activity, we chose to determine the structure of peptide L4-3pA(II) in solution using NMR techniques. A HPLC pure sample of peptide L4-3pA(II) was lyophilised then taken up in 550 ml of 10%$^2H_20$/90%H_20 and the pH adjusted to 5.3. The solution was then transferred into a 5 mm NMR tube. NMR spectra were acquired at 400 MHz on a Varian Inova 400 MHz NMR spectrometer. One-dimensional ^1H spectra were acquired with a sweep-width of 4000 Hz over 8K points.

Solvent suppression was achieved with selective low-power presaturation. Spectra were acquired at a series of sample temperatures (30°C, 15°C and 5°C) to check for temperature dependence of the peptide spectrum. The peptide did not show significant temperature dependence. All subsequent spectra were recorded at 30°C.

A series of 2D ^1H spectra were then recorded for L4-3pA. Typically, each spectrum was acquired with a sweep width of 4000 Hz over 1024 points, with 800 t_1, increments. TOCSY and DQF-COSY spectra were acquired for use in spin system assignments, while ROESY spectra were acquired to g nerate distance constraints. Spectra were initially

transformed using the Varian VNMR software package to check for the quality of the data. Subsequently, spectra were transformed using NMRpipe, and analysed using NMRview. Complete assignment of all non-exchangeable proton resonances was made. Dihedral constraints for the backbone f angles were derived from the $J^3_{NH-C\alpha H}$ coupling constants measured from 1D spectra. A total of 61 structurally important distance constraints and 3 backbone f angle constraints were determined from the NMR data for L4-3pA(II).

Structure calculation was carried out using the software package DYANA. Cyclisation of the peptide was achieved by introducing a set of special distance constraints to both bring the ends of the peptide together, and restrain the peptide bond angle to 180°. A modified version of the residue library containing a set of parameters defining a DPro residue was produced to allow calculation to include the DPro residue. A total of 100 structures were calculated on the basis of the NMR-derived constraint list by 10,000 steps of simulated annealing followed by 2,000 steps of minimisation of the DYANA target function. The 20 structures with the lowest target function were then selected as the final family of structures for the peptide. An overlay of these structures of peptide L4-3pA(II) can be found in Figure 18.

As can be seen in Figure 18, the conformation of the backbone of peptide L4-3pA(II) is uniquely defined in solution. In addition, side chain of Lys^4 adopts a single conformation up to its gamma-carbon atom, while the conformation of the side chain of Arg^5 is uniquely defined to the delta-nitrogen. The presence of a single backbone conformation and well-defined side chains for peptide L4-3pA is consistent with the biological data showing that compounds of closely related sequence to L4-3pA show either markedly reduced, or no neuronal survival activity in cell culture experiments. This exceptionally well-defined conformation of L4-3pA will be used as a template for the

design of non-peptidic molecules with neuronal survival promoting activity.

Example 26 Effect of Peptide L4-3pA(II) on Lesion-Induced Neurodegeneration *In Vivo*

The ability of peptide L4-3pA(II) to prevent or slow neurodegeneration *in vivo* was tested in a model of peripheral nerve lesion. To do this, newborn (24-48 hrs) Wistar rat pups (4 per treatment group) were rendered unconscious by ice-induced hypothermia. The median and ulnar nerve in the right forelimb was exposed, transected and wrapped with a piece of gel foam containing 10 µl a solution in PBS of L4-3pA(II) at one of two doses (10 µg/µl or 1 µg/µl) or PBS alone. Pups were re-united with their mothers and after 5 days were killed with a lethal injection of sodium pentobarbital (150 mg/kg) and perfused with a buffered 4% solution of paraformaldehyde. Spinal cords and DRGs were dissected out and embedded in paraffin, and serial transverse sections were cut, mounted on glass slides and stained with 0.5% cresyl violet. Neurons displaying prominent nucleoli were counted in every fifth section to include the entire rostrocaudal length of the DRG. Effects of peptides on neuronal loss were determined by comparing the number of neurons in the experimental side versus that in the intact contralateral side. Statistical comparisons between treatments was determined by one way ANOVA followed by post hoc Tukey's test.

As can be seen in Figure 19, both doses of peptide L4-3pA(II) significantly reduce the loss of of sensory (panel A: 100µg, 31 ±5% loss; 10µg, 23 ±3% loss) and motor (panel B: 100µg, 16 ±2% loss; 10µg, 11 ±6% loss) neurons that would otherwise die (panel A: sensory neurons 45 ±2% loss; panel B: motor neurons 35 ±2% loss) as a result of the lesion. The degree of rescue is similar to that seen with other neurotrophic factors, such as LIF (Cheema *et al* 1994a; 1994b). It is worthy of note that the best rescue of both sensory and motor neurons was obtained

with the smaller dose (10 µg) of L4-3pA(II). This may reflect the nature of the apparently bell-shaped concentration response curve we have observed for L4-3pA(II).

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing specification.

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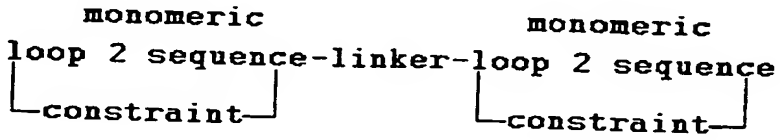
CLAIMS:

1. A cyclic compound comprising one or more cyclic moieties, which has a biological activity of brain-derived neurotrophic factor (BDNF).

2. A compound according to claim 1, wherein the compound is monocyclic monomeric, bicyclic dimeric, or tricyclic dimeric, ~~as described herein.~~

3. A compound according to claim 2, wherein the compound is a bicyclic dimeric compound of general formula (I):

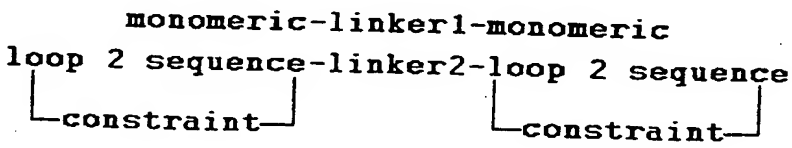


(I).

4. A compound according to claim 3, wherein the constraint comprises a covalent grouping of atoms.

5. A compound according to claim 4, wherein the constraint and the linker may be the same or different.

6. A compound according to claim 2, wherein said compound is a tricyclic dimeric compound of general formula (II):



(II).

7. A compound according to claim 6, wh rein each of the constraint, link r 1 and linker 2 may be the same or

different.

8. A compound according to any one of claims 3 to 7, wherein each of the constraint, linker, linker 1 or linker 2 has between at 0 to 20 carbon atoms, and 0 to 10 heteroatoms, wherein said heteroatoms are selected from the group consisting of N, O, S, and P.

9. A compound according to claim 8, wherein each of the constraint, linker, linker 1 or linker 2, is either a straight or branched chain containing either saturated, unsaturated and/or aromatic rings.

10. A compound according to claim 8 or claim 9, wherein each of the constraint, linker, linker 1 or linker 2, comprises single and/or double bonds.

11. A compound according to according to any one of claims 8 to 10,wherein each of the constraint, linker, linker 1 or linker 2, comprises one or more chemical groups selected from the group consisting of amide, ester, disulphide, thioether, ether, phosphate and amine.

12. A compound according to any one of claims 3 to 10, wherein the constraint is obtained by either:

(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group; or

(ii) cyclising via the formation of a covalent bond between the side chains of two residues, either directly or via a spacer group as described in (i) above; or

(iii)a disulphide bond between two cysteine residues, either directly or via a spacer group as described in (i) abov ; or

(iv) a thio ther bond between a cysteine residue

and an ω-halogenated amino acid residu , either directly or via a spacer group as described in (i) above; or

(v) cyclising via the formation of an amide bond between a side chain and either the C-terminal carboxyl or N-terminal amine, either directly or via a spacer group as described in (i) above.

13. A compound according to any one of claims 3 to 10, wherein each of the linker, linker 1 or linker 2 is obtained by either:

(i) cyclising via the formation of a covalent bond between the side chains of two residues, either directly or via a spacer group; or

(ii) a disulphide bond between two cysteine residues, either directly or via a spacer group as described in (i) above; or

(iii) a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above; or

(iv) cyclising via the formation of an amide bond between a side chain and either the C-terminal carboxyl or N-terminal amine, either directly or via a spacer group as described in (i) above.

14. A compound according to claim 12 or claim 13, wherein said formation of a covalent bond between the side chains of two residues is via the formation of an amide bond between a lysine residue and either an aspartic acid or glutamic acid residue.

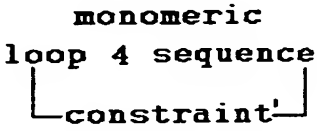
15. A compound according to claim 12 or claim 13, wherein the side chain in (ii) is either a lysine or an aspartate residue.

16. A compound according to claim 12, wherein the cyclising of the N-terminal amine with the C-terminal carboxyl acid is via condensation with an ω-amino

carboxylic acid.

17. A compound according to any one of claims 12 to 16, wherein the residues contributing to the side chains are either derived from the monomeric loop 2 sequence itself, or incorporated into or added on to the monomeric loop 2 sequence.

18. A compound according to claim 2, wherein said compound is a monomeric, monocyclic compound of general formula (III):



(III).

19. A compound according to claim 17, wherein said constraint is obtained by cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group.

20. A compound according to claim 19, wherein the spacer group consists of one or more additional amino acid residues.

21. A compound according to claim 20, wherein the one or more additional amino acid residues includes α- and ω-amino carboxylic acid residues.

22. A compound according to claim 20, wherein the residues contributing the side chains are derived from the monomeric loop 4 sequence itself, or incorporated into or added on to the monomeric loop 4 sequence.

23. A compound according to any on of claims 1 to 22, wherein one or more amino acids is replaced by its

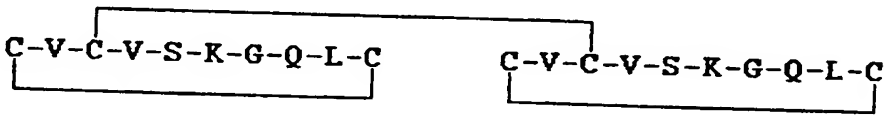
corresponding D-amino acid.

24. A compound according to any one of claims 1 to 23, wherein one or more peptide bonds is replaced by a structure more resistant to metabolic degradation.

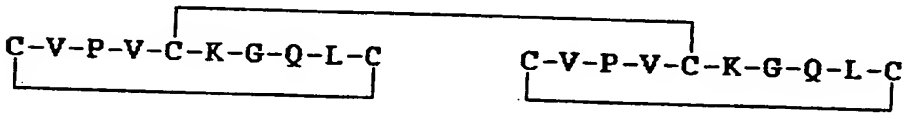
25. A compound according to any one of claims 1 to 23, wherein individual amino acids in said compound are replaced by analogous structures as described herein.

26. A compound according to claim 25, wherein said analogous structures are selected from the group consisting of *gem*-diaminoalkyl groups, alkylmalonyl groups (with or without modified termini), alkyl, acyl and amine groups.

27. A compound according to claim 1, wherein said compound is of formula (IV) or formula (V):

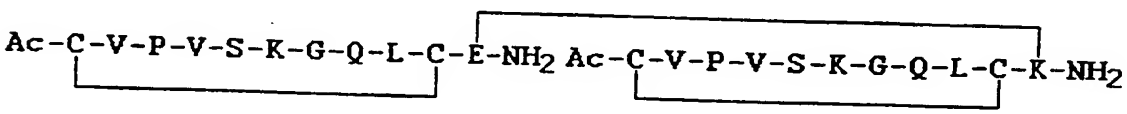


(IV)



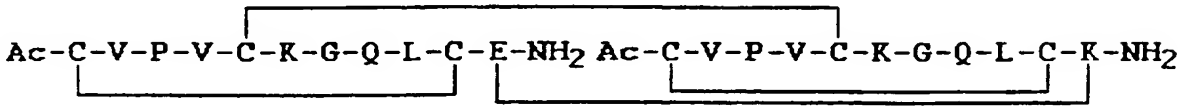
(V).

28. A compound according to claim 1 wherein said compound is of formula (VI):



(VI).

29. A compound according to claim 1, wherein said compound is of formula (VII):



(VII).

30. A compound according to claim 1, wherein said compound is of formula (VIII):

(VIII).

31. A composition, comprising a compound according to any one claims 1 to 30, together with a pharmaceutically-acceptable carrier, or a carrier or diluent which does not adversely affect the growth of cells in culture.

32. A composition according to claim 30, wherein said composition is formulated for oral, intravenous, subcutaneous, intramuscular, intrathecal, intraventricular or topical administration.

33. A composition according to claim 31 or claim 32, wherein the carrier is selected from the group consisting of dextrose, mannitol, sucrose, and lactose.

34. A composition according to claim 33, further comprising one or more buffer and/or bulking agents.

35. A composition according to claim 34, wherein the buffer is selected from the group consisting of acetate,

citrate and phosphate.

36. A composition according to claim 34, wherein the bulking agent is selected from the group consisting of serum albumin and human serum albumin.

37. A composition according to claim 31, used as a culture medium additive for promotion of growth of neuronal cells *in vitro*.

38. A composition according to claim 37, wherein the carrier or diluent is water, a saline solution, or a buffer solution.

39. A composition according to claim 37 or claim 38, wherein the concentration of compound is in the range 1-500μM.

40. A culture medium according to claim 39, wherein the concentration of compound is in the range 1-100μM.

41. A method of treating a condition characterised by neuronal deficit or neuronal death, comprising the step of administering an effective amount of a compound according to any one of claims 1 to 30, or a composition according to any one of claims 31 to 37, to a subject in need of such treatment.

42. A method according to claim 41, wherein the condition being treated is selected from the group consisting of neurodegenerative diseases, neurodegenerative conditions caused by insult, and peripheral sensory neuropathies.

43. A method according to claim 42, wherein the neurodegenerative diseases are selected from the group consisting of motor neurone disease (amyotrophic lateral

sclerosis), progr ssive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrom , Guamanian Parkinsonian dementia complex, progressive bulbar atrophy and Alzheimer's disease.

44. A method according to claim 42, wherein the insult arises from ischaemia, hypoxia, neural injury, surgery, and exposure to neurotoxins such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

45. A method according to claim 42, wherein the peripheral sensory neuropathies result from exposure to drugs (such as cis-platin), toxins, diabetes and mononeuropathy multiplex.

46. A method according to claim 41, wherein the route of administration is selected from the group consisting of oral, intravenous, subcutaneous, intramuscular, intrathecal, intraventricular and topical.

47. Use of a compound according to any one of claims 1 to 30, or a composition according to any one of claims 31 to 37 in the manufacturer of a medicament used for treating a condition characterised by neuronal deficit or neuronal death.

48. Use according to claim 47, wherein the condition being treated is selected from the group consisting of neurodegenerative diseases, neurodegenerative conditions caused by insult, and peripheral sensory neuropathies.

49. Use according to claim 48, wherein the neurodegenerative diseases are selected from the group consisting of motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth diseas , Parkinson's

Disease, Parkinson-Plus syndrome, Guamanian Parkinsonian dementia complex, progressive bulbar atrophy and Alzheimer's disease.

50. Use according to claim 48, wherein the insult arises from ischaemia, hypoxia, neural injury, surgery, and exposure to neurotoxins such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

51. Use according to claim 48, wherein the peripheral sensory neuropathies result from exposure to drugs (such as cis-platin), toxins, diabetes and mononeuropathy multiplex.

52. Use according to claim 47, wherein the route of administration is selected from the group consisting of oral, intravenous, subcutaneous, intramuscular, intrathecal, intraventricular and topical.

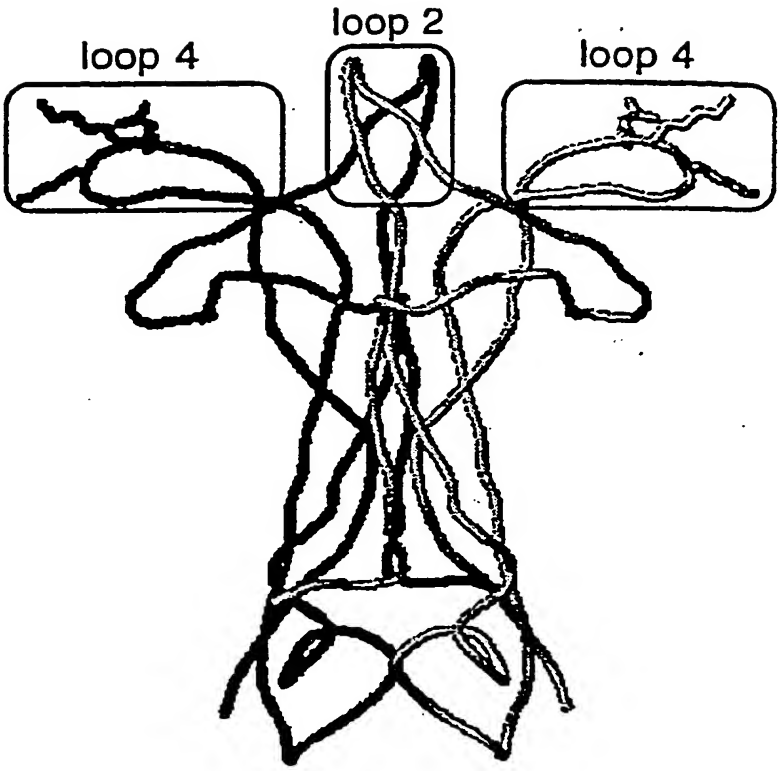


FIGURE 1

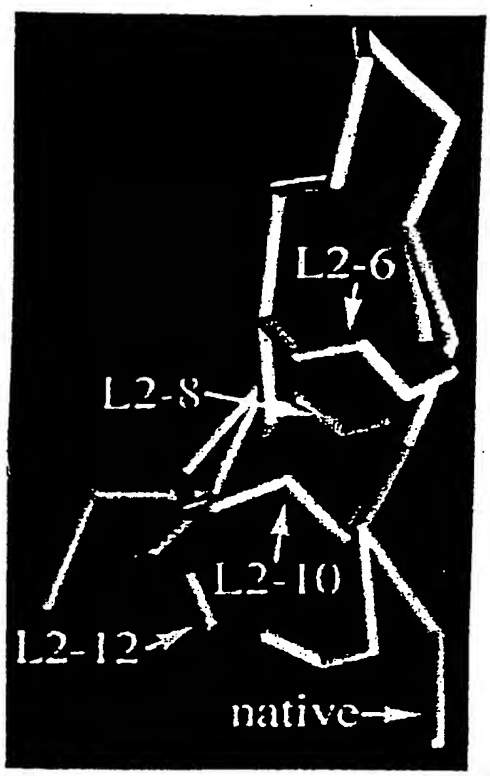


FIGURE 2

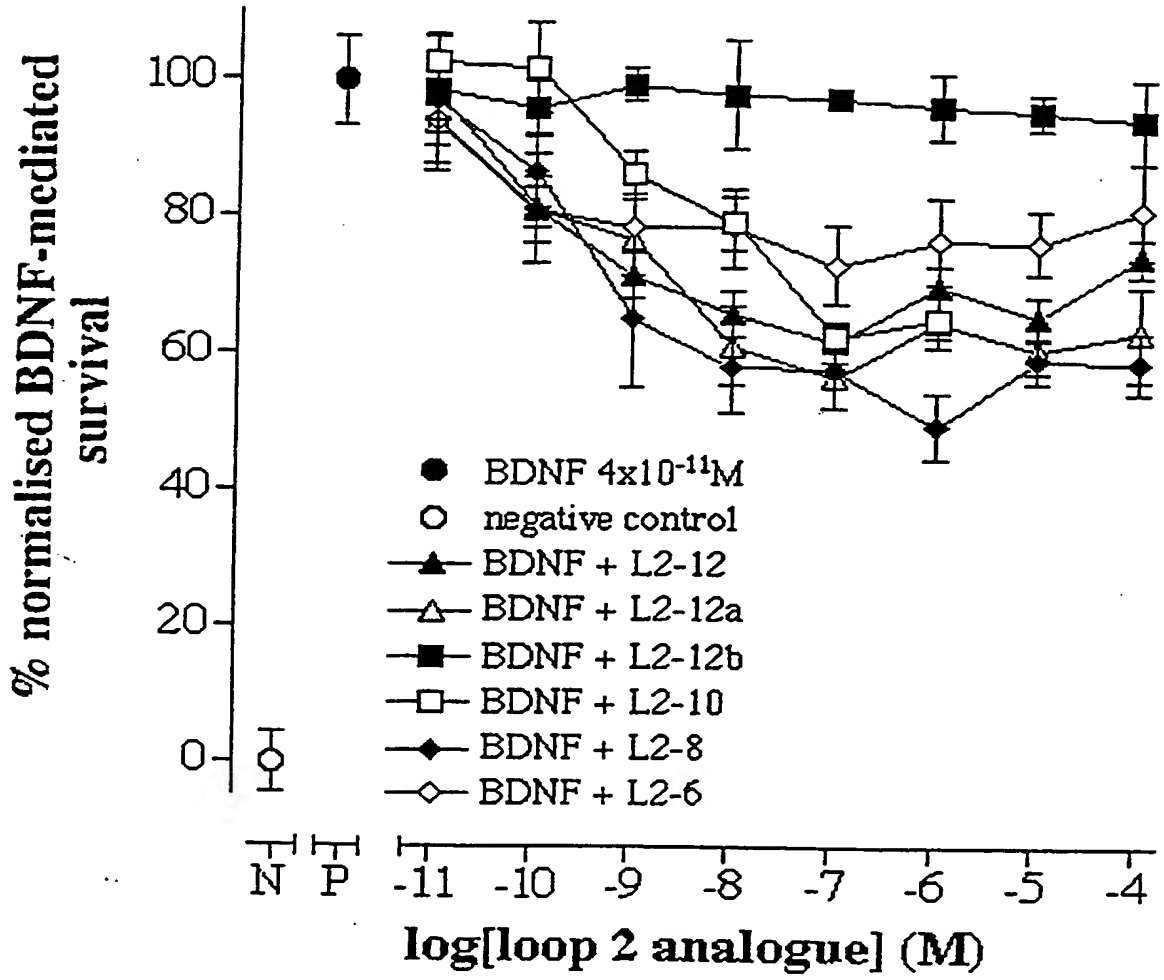

% normalised BDNF-mediated survival
100
80
60
40
20
0
BDNF 4x10-11M
negative control
BDNF + L2-12
BDNF + L2-12a
BDNF + L2-12b
BDNF + L2-10
BDNF + L2-8
BDNF + L2-6
N
P
-11
-10
-9
-8
-7
-6
-5
-4
log[loop 2 analogue] (M)

FIGURE 3

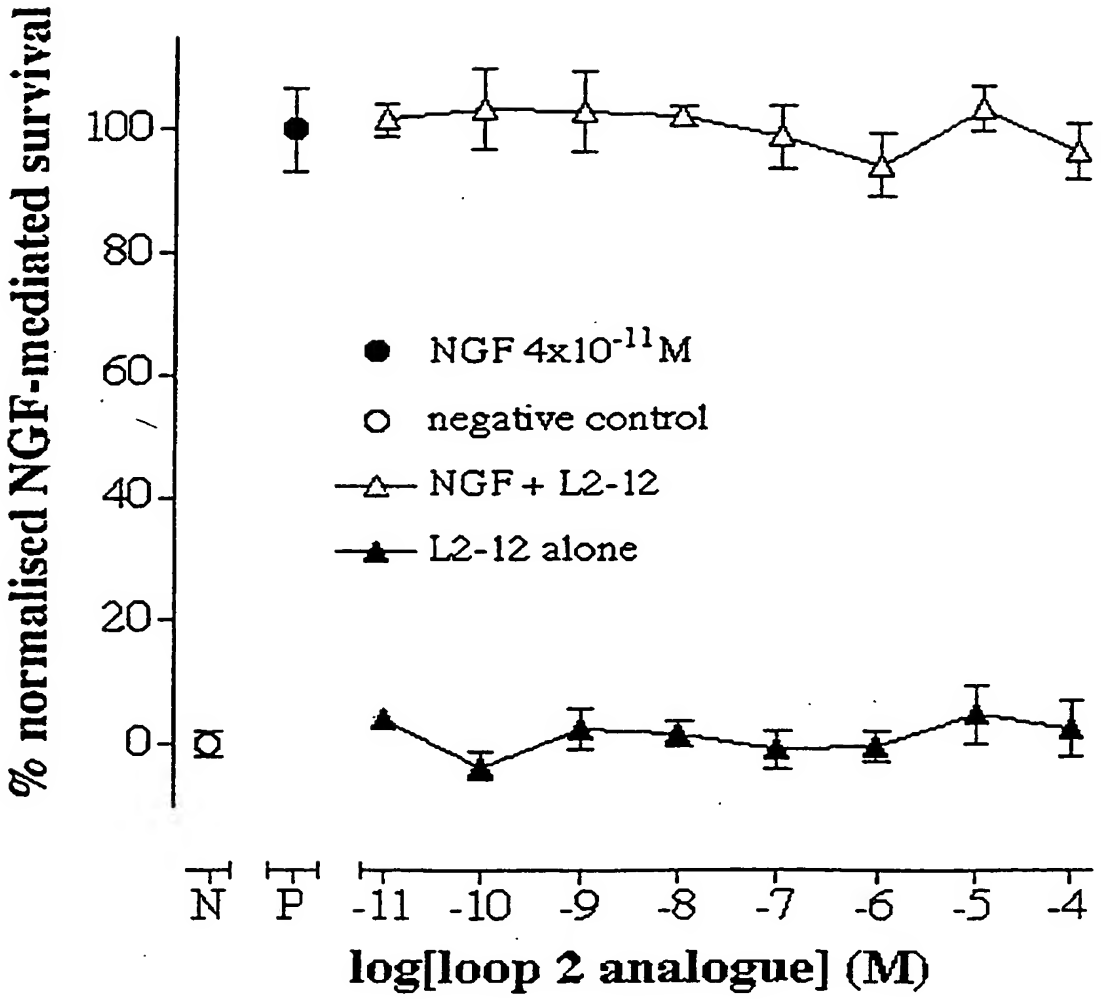

% normalised NGF-mediated survival
100
80
60
40
20
0
NGF 4x10-11M
negative control
NGF + L2-12
L2-12 alone
N
P
-11
-10
-9
-8
-7
-6
-5
-4
log[loop 2 analogue] (M)

FIGURE 4

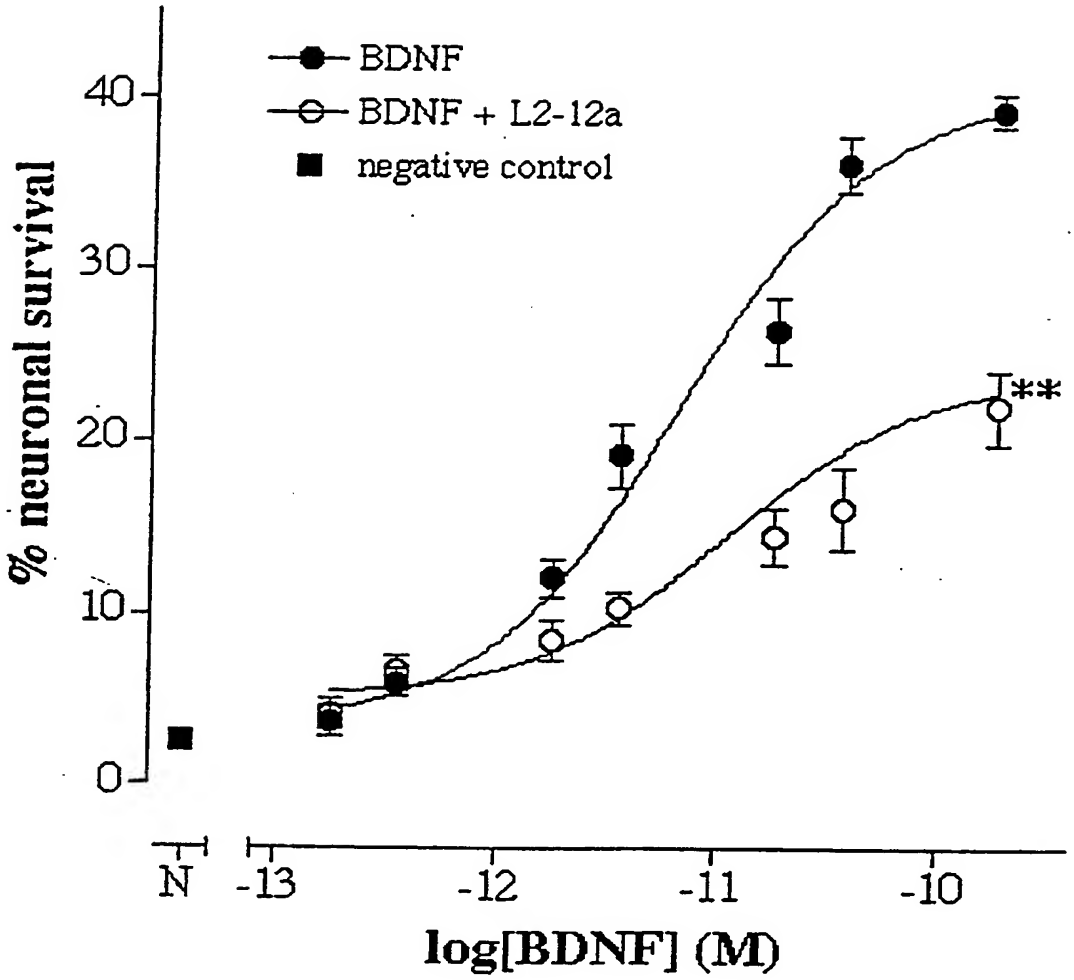

BDNF
BDNF + L2-12a
negative control
% neuronal survival
40
30
20
10
0
**
N
-13
-12
-11
-10
log[BDNF] (M)

FIGURE 5

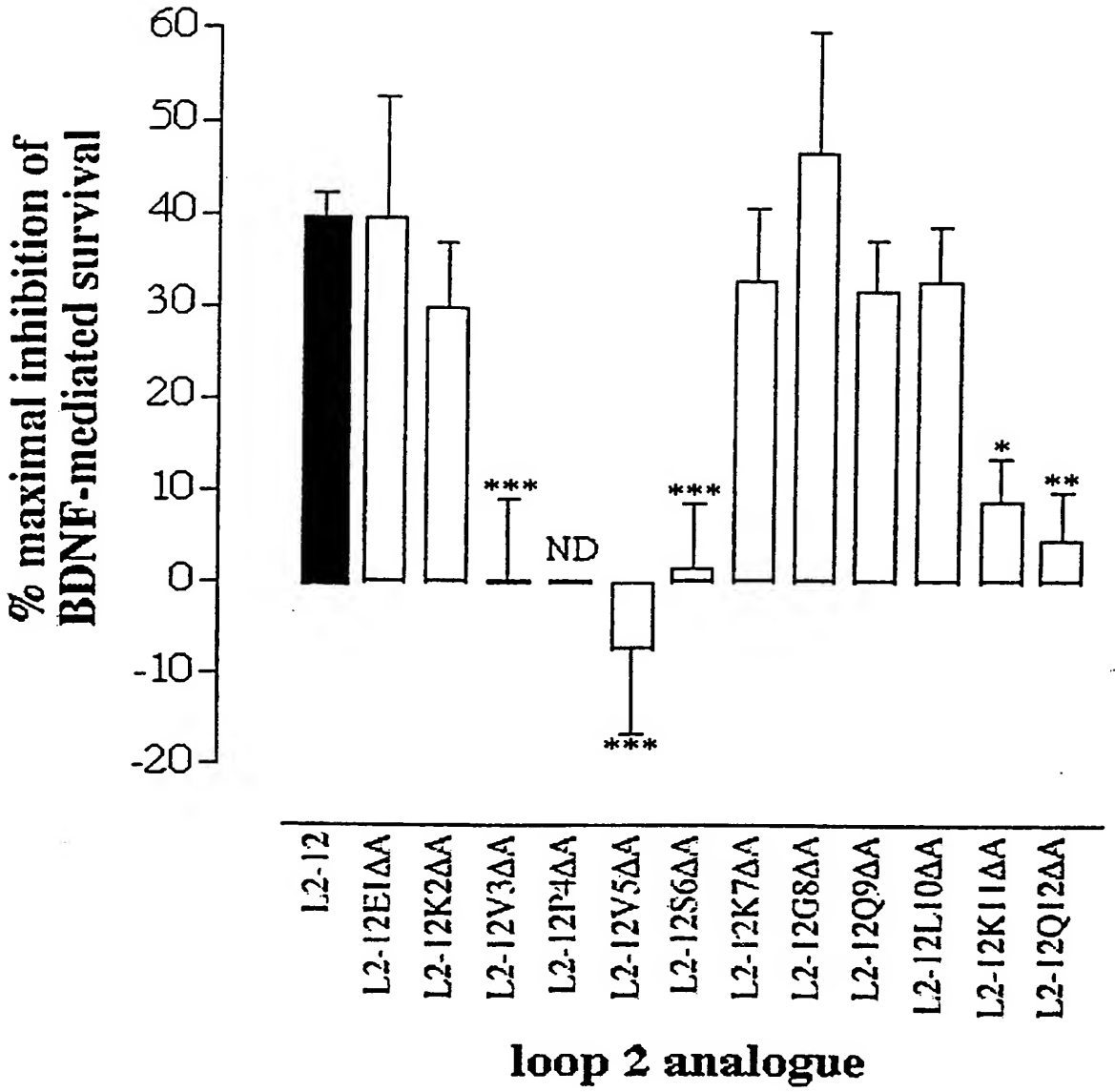

% maximal inhibition of BDNF-mediated survival
60
50
40
30
20
10
0
-10
-20
***
ND
***
***
*
**
L2-12
L2-12E1ΔA
L2-12K2ΔA
L2-12V3ΔA
L2-12P4ΔA
L2-12V5ΔA
L2-12S6ΔA
L2-12K7ΔA
L2-12G8ΔA
L2-12Q9ΔA
L2-12L10ΔA
L2-12K11ΔA
L2-12Q12ΔA
loop 2 analogue

FIGURE 6

Schematic view of loop 2 region of BDNF

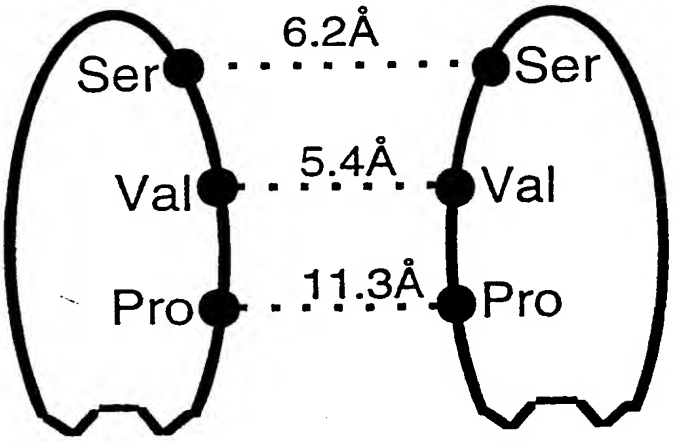
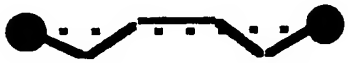


FIGURE 7A

Schematic view of disulphide bridge



average 5.3Å 90%CI 5.22-5.44Å

FIGURE 7B

FIGURE 7

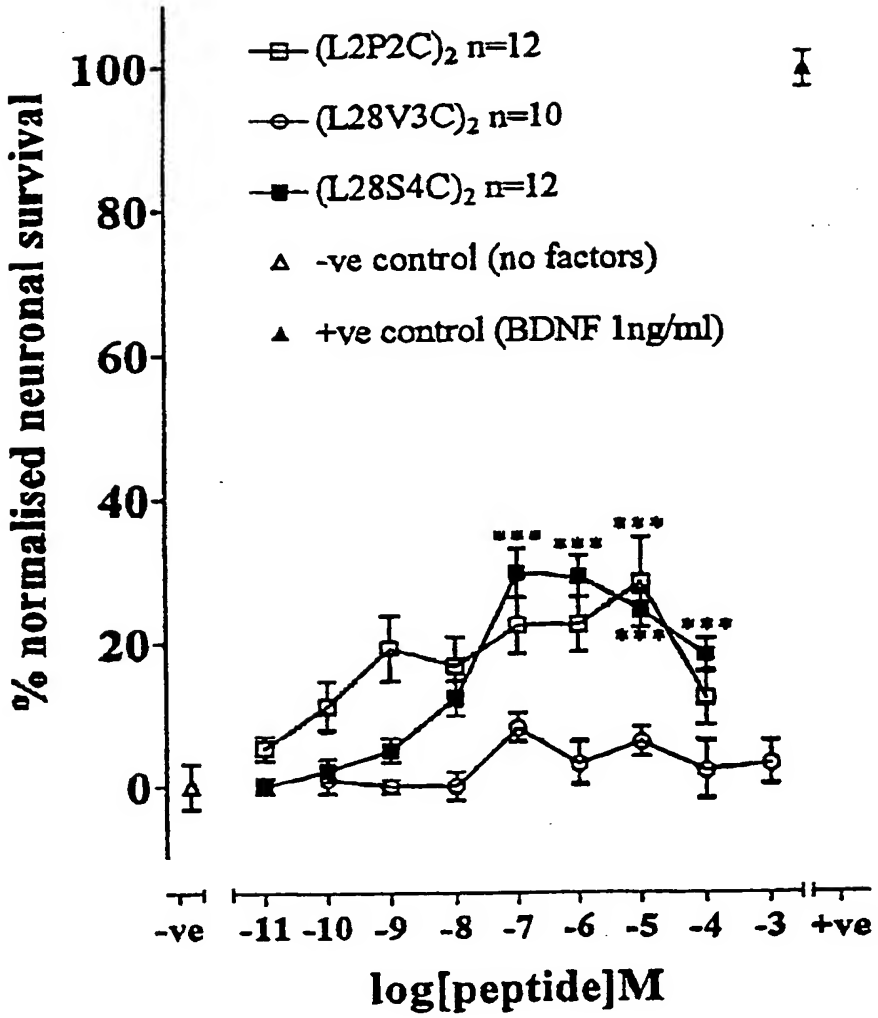

(L2P2C)2 n=12
(L28V3C)2 n=10
(L28S4C)2 n=12
-ve control (no factors)
+ve control (BDNF 1ng/ml)
% normalised neuronal survival
100
80
60
40
20
0
***
***
***
***
-ve
-11
-10
-9
-8
-7
-6
-5
-4
-3
+ve
log[peptide]M

FIGURE 8

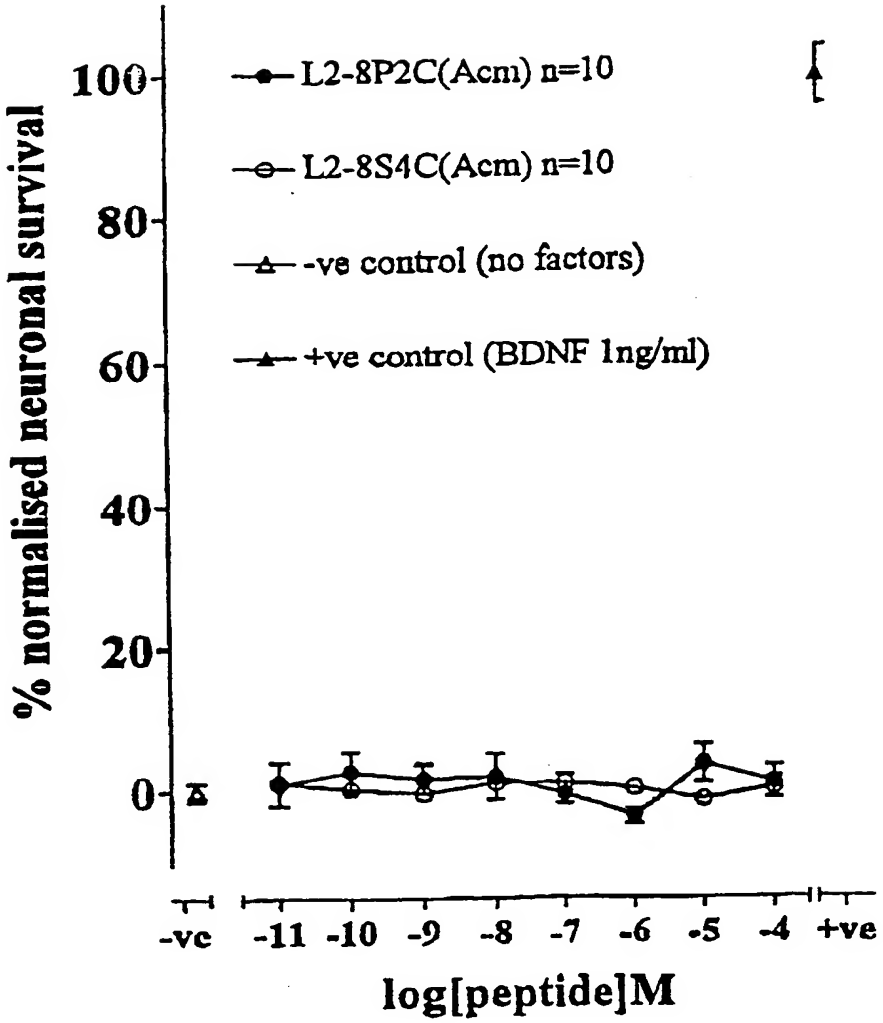

L2-8P2C(Acm) n=10
L2-8S4C(Acm) n=10
-ve control (no factors)
+ve control (BDNF 1ng/ml)
% normalised neuronal survival
100
80
60
40
20
0
-vc
-11
-10
-9
-8
-7
-6
-5
-4
+ve
log[peptide]M

FIGURE 9

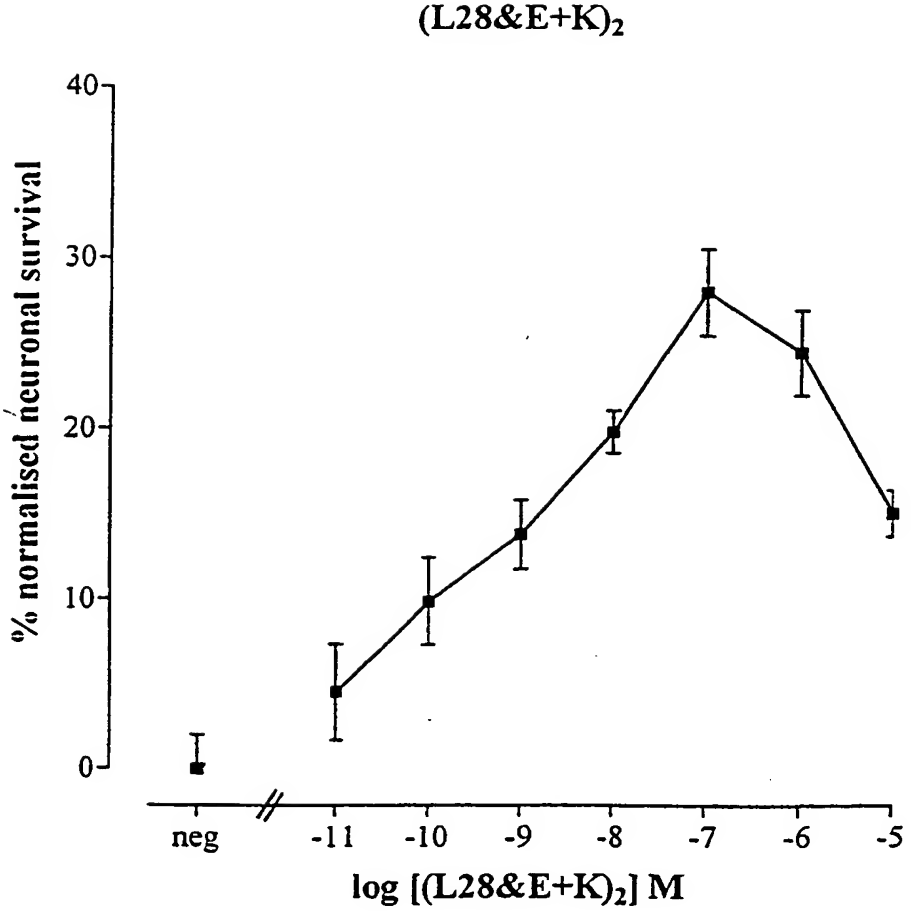

(L28&E+K)2
% normalised neuronal survival
40
30
20
10
0
neg
-11
-10
-9
-8
-7
-6
-5
log [(L28&E+K)2] M

FIGURE 10

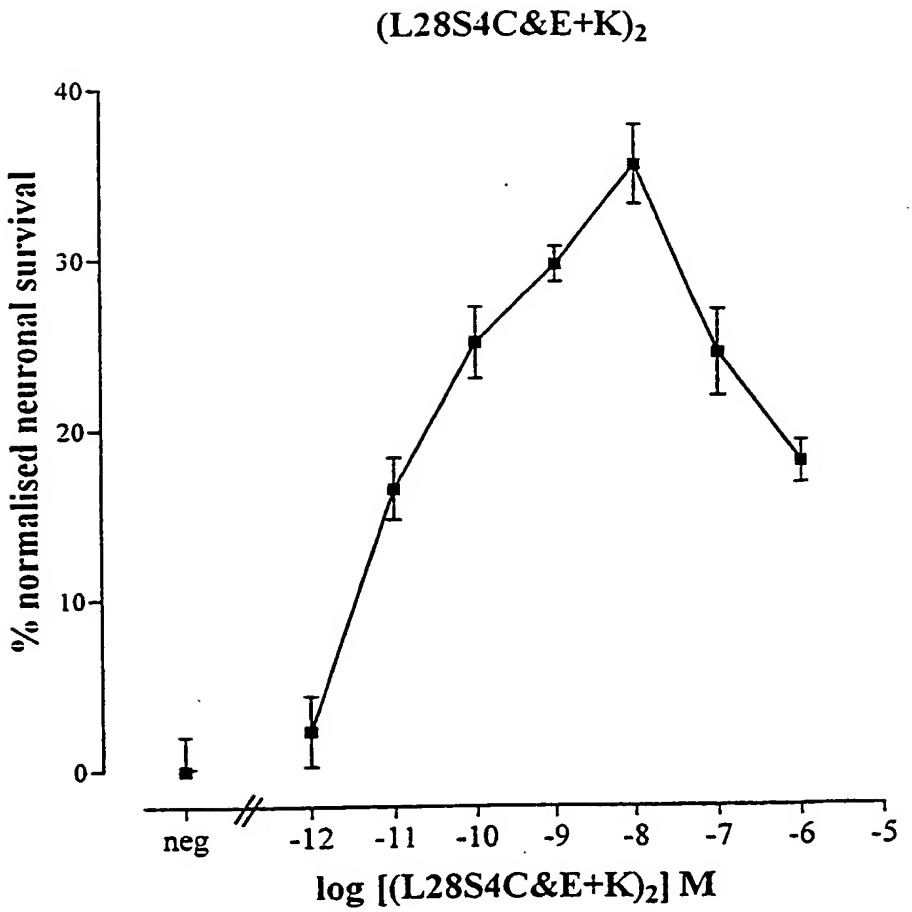

(L28S4C&E+K)2
% normalised neuronal survival
40
30
20
10
0
neg
-12
-11
-10
-9
-8
-7
-6
-5
log [(L28S4C&E+K)2] M

FIGURE 11

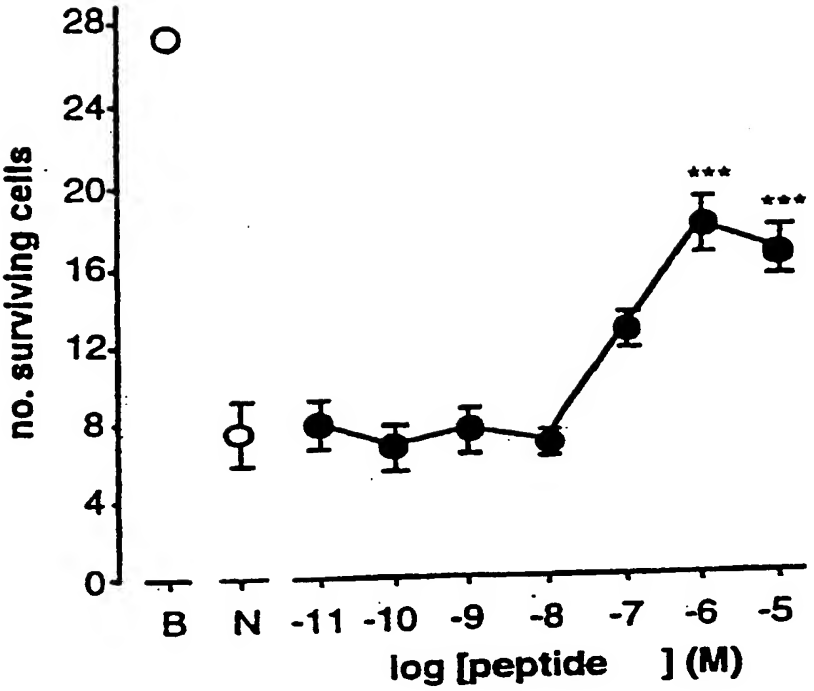

28
24
20
16
12
8
4
0
no. surviving cells
O
***
***
B
N
-11
-10
-9
-8
-7
-6
-5
log [peptide] (M)

FIGURE 12

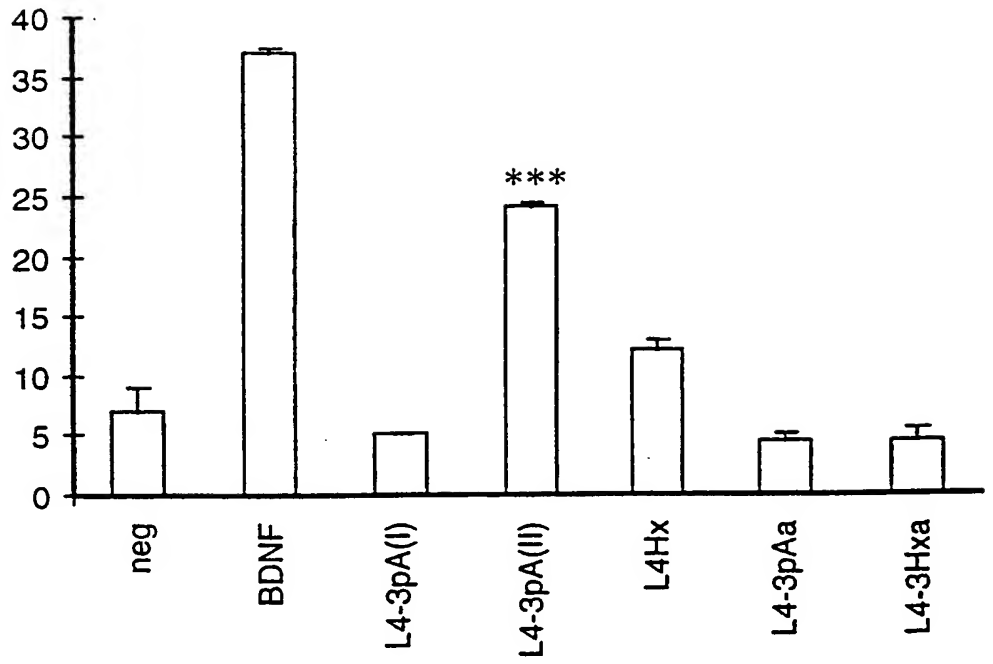

40
35
30
25
20
15
10
5
0
***
neg
BDNF
L4-3pA(I)
L4-3pA(II)
L4Hx
L4-3pAa
L4-3Hxa

FIGURE 13

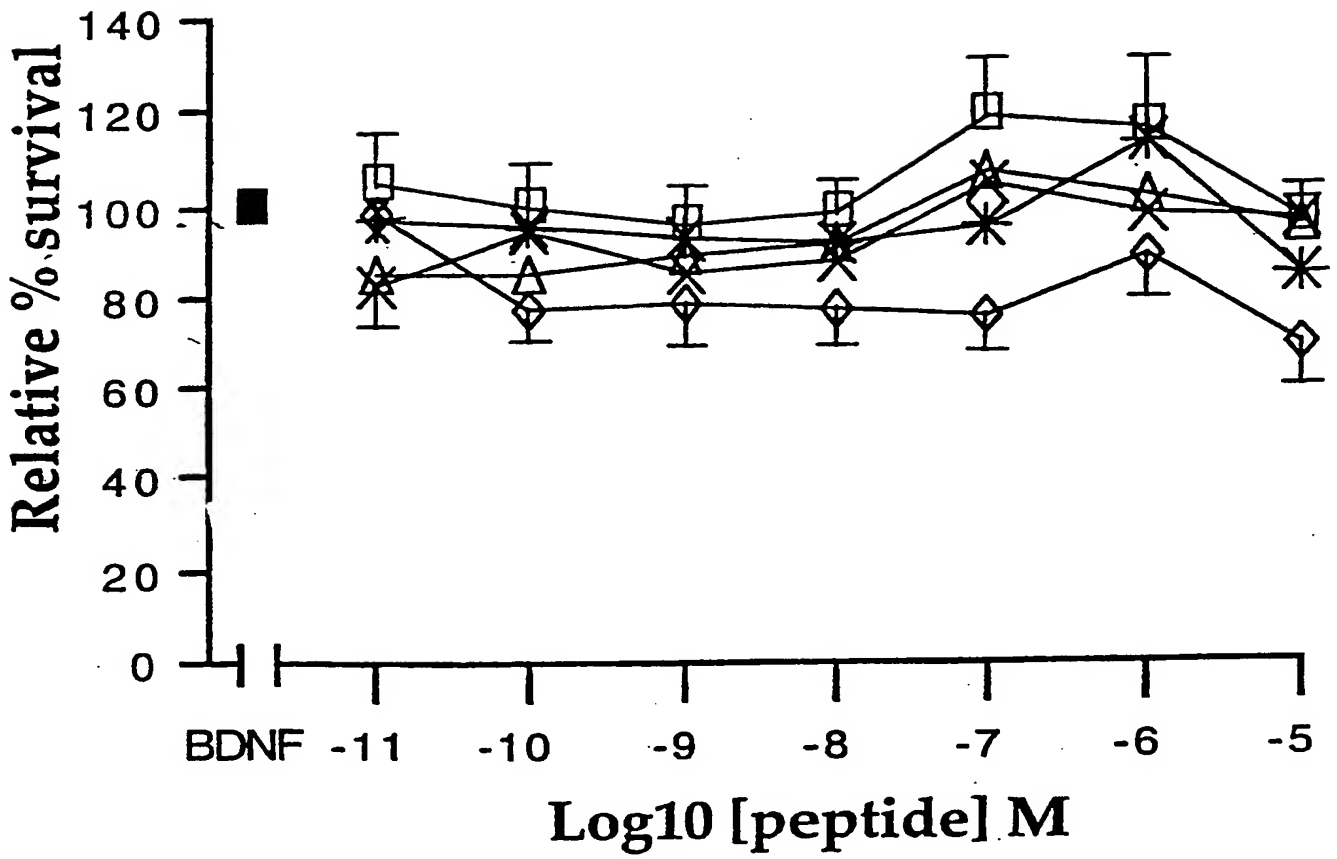

Relative % survival
140
120
100
80
60
40
20
0
BDNF
-11
-10
-9
-8
-7
-6
-5
Log10 [peptide] M

FIGURE 14

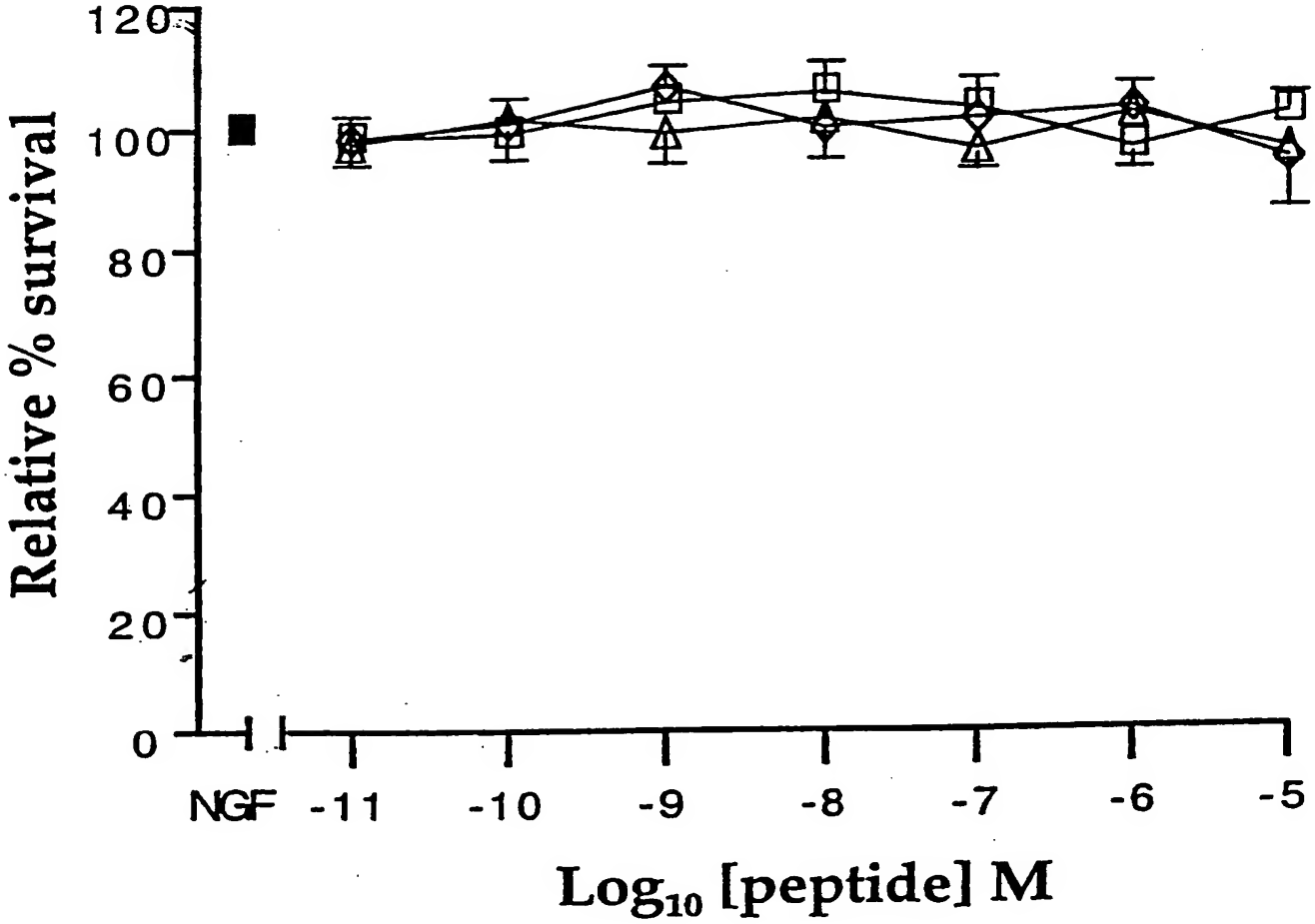

120
100
80
60
40
20
0
Relative % survival
NGF
-11
-10
-9
-8
-7
-6
-5
Log10 [peptide] M

FIGURE 15

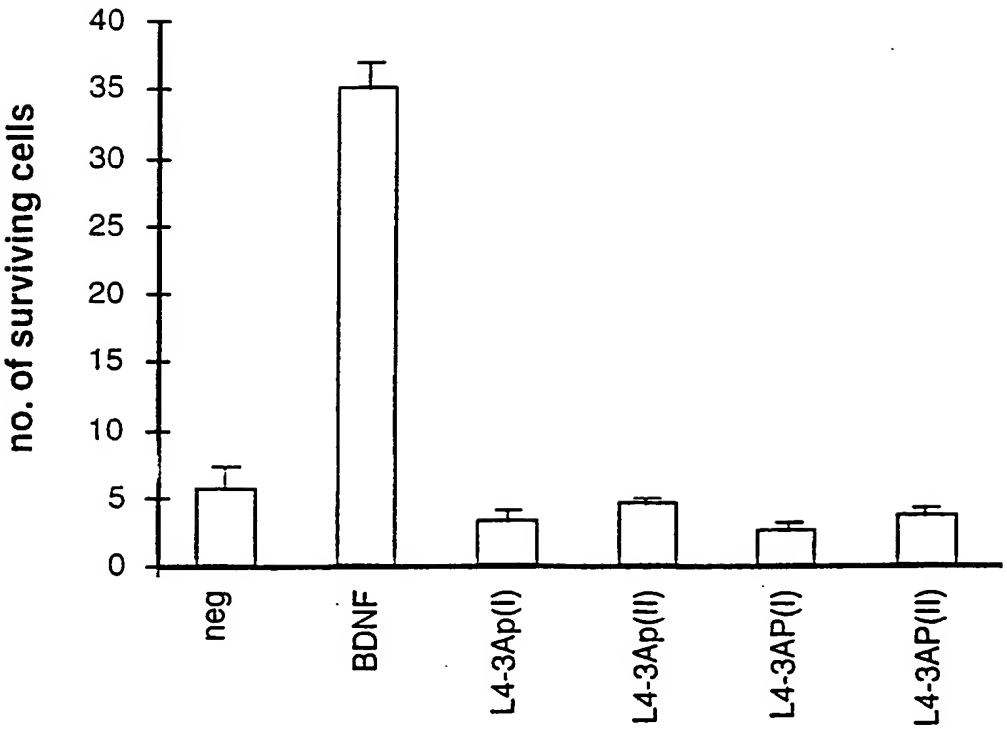

no. of surviving cells
40
35
30
25
20
15
10
5
0
neg
BDNF
L4-3Ap(I)
L4-3Ap(II)
L4-3AP(I)
L4-3AP(II)

FIGURE 16

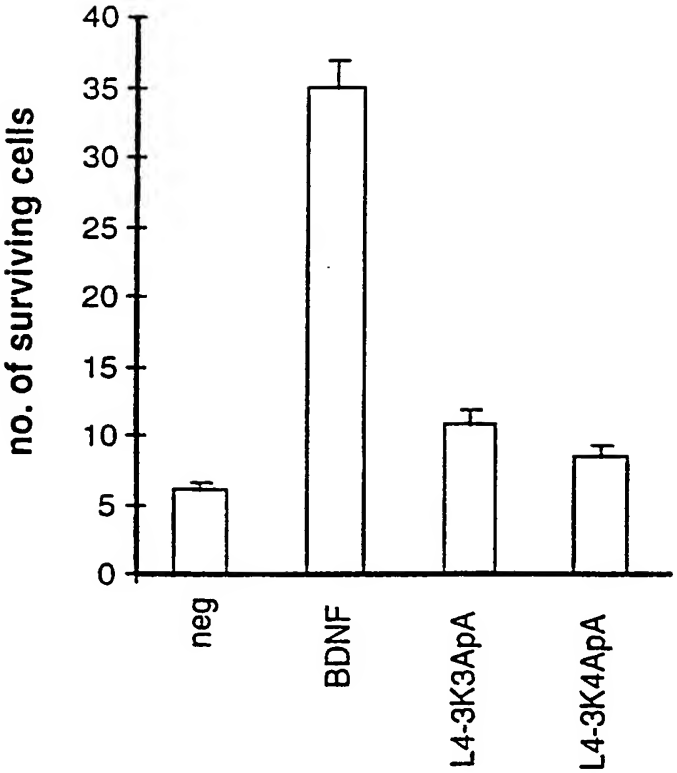

no. of surviving cells
40
35
30
25
20
15
10
5
0
neg
BDNF
L4-3K3ApA
L4-3K4ApA

FIGURE 17

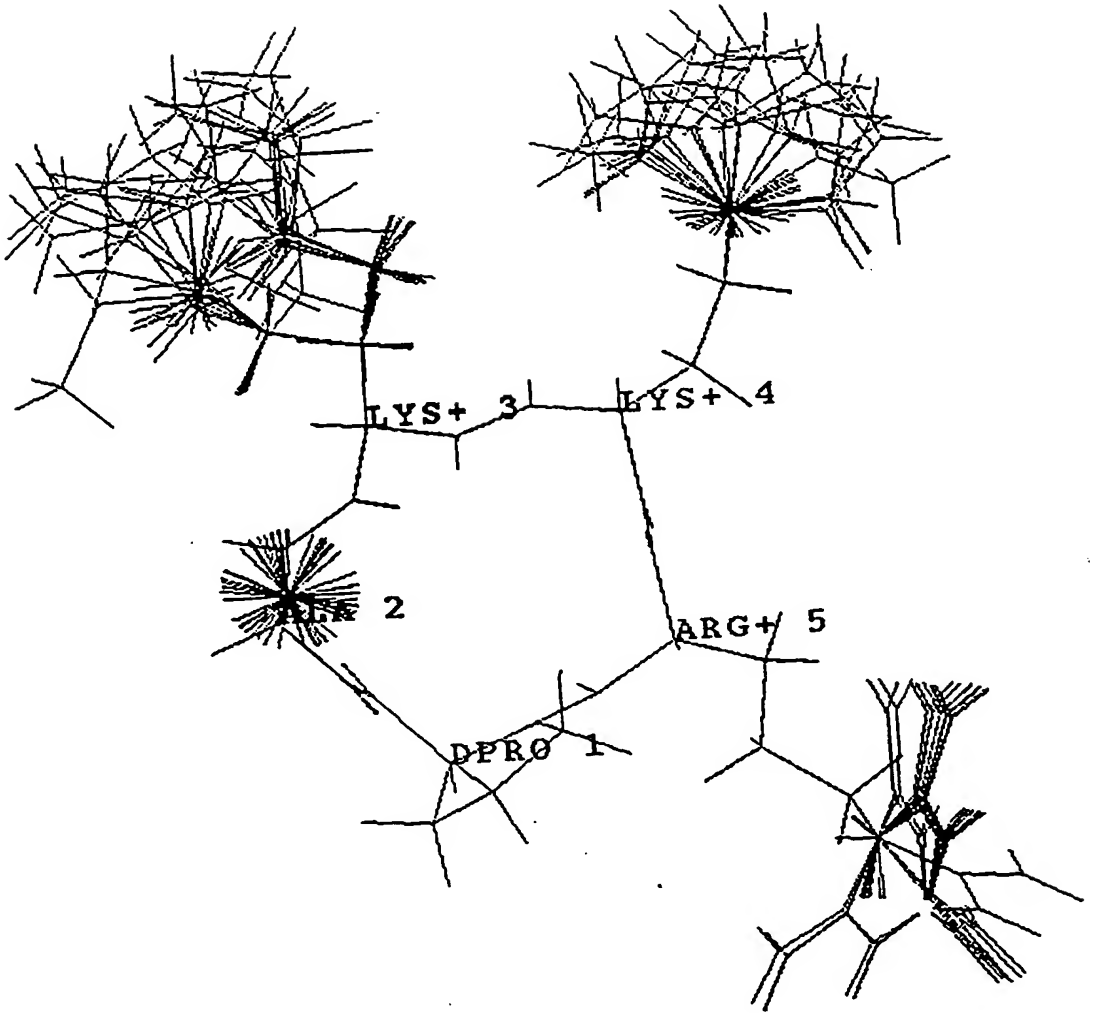


FIGURE 18

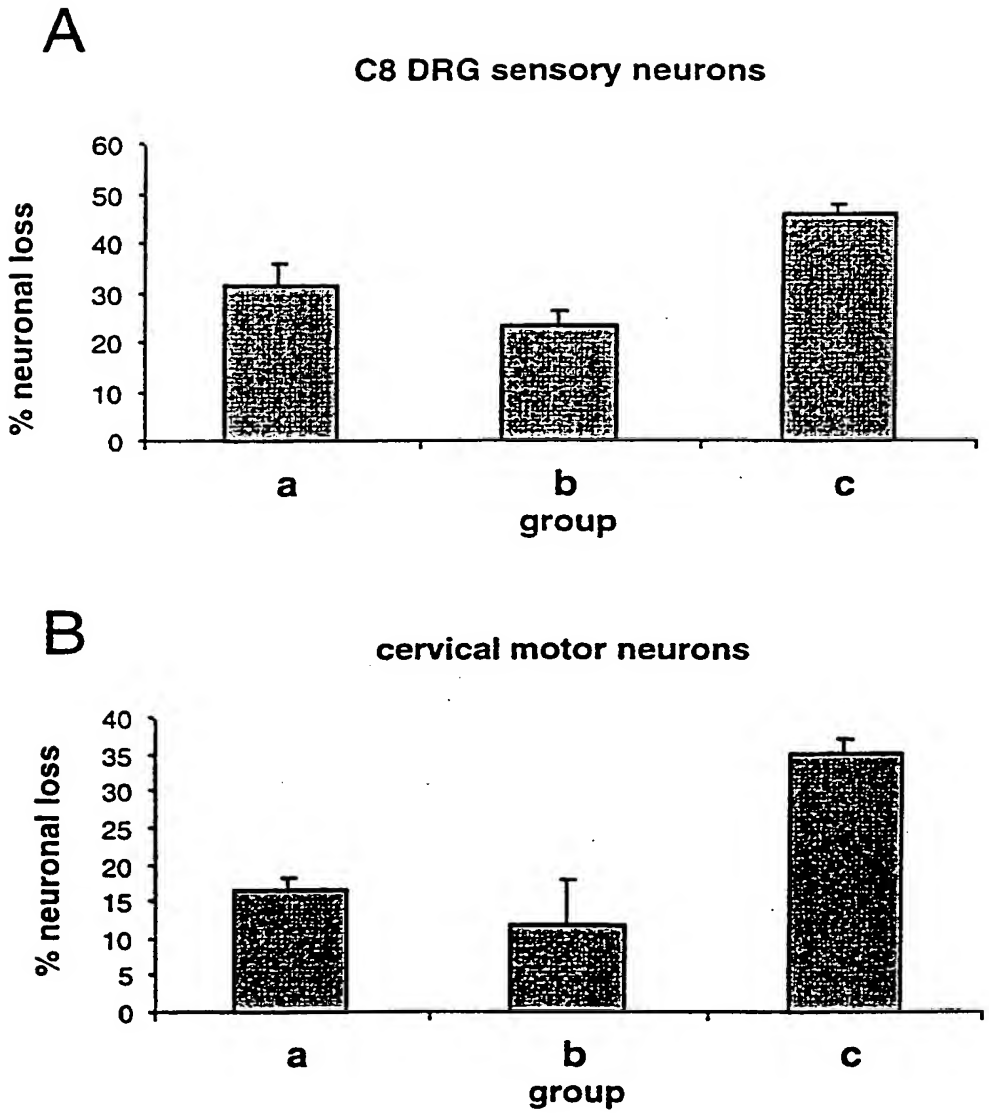

A
C8 DRG sensory neurons
% neuronal loss
60
50
40
30
20
10
0
a
b
c
group
B
cervical motor neurons
% neuronal loss
40
35
30
25
20
15
10
5
0
a
b
c
group

FIGURE 19

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